Vol. XXXVII July, 1948 No. 7

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JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

VOLUME XXXVII — *JULY, 1948* — NUMBER 7 — CONSECUTIVE NO. 14

Micro-Determination of Molybdenum and Tungsten in Organic Materials*

By C. F. BICKFORD, W. S. JONES, and J. S. KEENE†

Colorimetric methods for determining small amounts of tungsten and molybdenum, using "dithiol" (4-methyl-1,2-dimercaptobenzene), have been developed. Since both the tungsten and molybdenum compounds with "dithiol" have characteristic colors and impart them to butyl acetate upon solution, a spectrophotometer may be conveniently used in the estimation of the two metals. The methods are satisfactory for the range 0 to 50 micrograms in an individual test. The metals may be determined in the presence of each other, since citric acid prevents the precipitation of tungsten while permitting the precipitation of molybdenum with "dithiol." The methods have been developed for organic material, but the adaptation for inorganic material would be quite simple.

PHOSPHOTUNGSTIC and phosphomolybdic acids are commonly used for the precipitation of organic compounds; the classic example being the precipitation of alkaloids. It is important to know the amounts of tungsten and molybdenum remaining in the final products which are eventually to be used as drugs. The determination of small amounts of tungsten and molybdenum in the range 0 to 50 micrograms may be conveniently made using the reagent 4-methyl-1,2-dimercaptobenzene (1). This reagent, also designated as "dithiol," has been used as a specific reagent for stannous tin (2, 3) for which purpose it is very satisfactory. Many common metals, such as lead, copper, and bismuth, form insoluble precipitates with dithiol. These precipitates are often highly colored. Some of them, however, are black. The stannous compound is bright red, and it is this characteristic color which makes dithiol an excellent reagent for tin. A compound which is formed with a metal and dithiol will be designated as a "metal-dithiol" in this article i.e., tungsten-dithiol, molybdenum-dithiol, etc. These compounds are more properly termed mercaptides (4).

METAL-DITHIOLS

When conditions are suitable, dithiol forms precipitates with molybdenum (molybdate) and tungsten (tungstate) which are insoluble in dilute acid solutions and which have intense and characteristic colors. However, the important factor in the use of dithiol for the colorimetric estimation of the two metals is the ability of each dithiol precipitate to dissolve readily in butyl acetate to yield intensely colored solutions, which are reasonably stable (5). The color of the tungsten-dithiol in butyl acetate is blue-green; and the color of the molybdenum-dithiol is yellow-green (6).

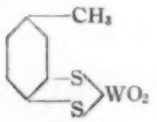
As little as 5 μg. of either element, dissolved as its dithiol compound in butyl acetate, may be easily de-

* Received December 5, 1947, from the Pharmaceutical Development Division, E. R. Squibb and Sons, Brooklyn, N. Y. Presented to the Scientific Section, A. PH. A., Milwaukee meeting, August, 1947.

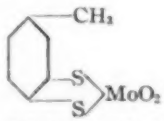
† Appreciation is extended to F. O. Howland and A. T. Warren, Control Laboratory, E. R. Squibb and Sons, Brooklyn, for the spectrophotometric data, relating to metal-dithiol compounds in butyl acetate, presented in this article.

tected and measured in a volume of 25 cc. In addition, the extraction of the precipitate from the aqueous mixture with butyl acetate tends to separate the two elements from other metal-dithiols, which are insoluble in butyl acetate and which tend to collect at the interface of the water and butyl acetate layers. An example of such a metal-dithiol is cupric-dithiol. The stannous-dithiol dissolves in some organic solvents with the loss of its characteristic bright red color, the resulting solutions having in contrast only a faintly perceptible yellow color.

Tungsten and molybdenum appear to be present as the tungstate and the molybdate in the solutions as prepared for testing. No attempt has been made to learn the exact nature of the dithiol precipitates. The compounds potassium dioxydisulfotungstate ($K_2WO_2S_2.H_2O$) and potassium dioxydisulfomolybdate ($K_2MoO_2S_2$) are described in the literature (7). By analogy, the following structures are suggested for tungsten-dithiol and molybdenum-dithiol.



Tungsten-dithiol



Molybdenum-dithiol

Stannous chloride and thiocyanate have been used for the estimation of small amounts of tungsten (8). However, the many determinations which have been made in the laboratory indicate that dithiol is a better reagent to use in estimating tungsten, both because of the characteristic intense color and because of the stability of this color in butyl acetate.

Measurements of the color intensity with the photometer are easily made. A combination of filters with optimum transmission, 610 to 660 mμ, is used. The maximum transmission is at about 630 mμ. The same photometer arrangement serves for the measurement of molybdenum-dithiol and tungsten-dithiol colors, an advantage when both elements are present together. Although the colors closely resemble each other, they are easily distinguished after a little experience.

Since many other elements give insoluble precipitates with dithiol, a method for removing them before testing for molybdenum and tungsten is desirable. Dithizone (diphenylthiocarbazone) is an excellent reagent to remove many of the interfering metals and may be used routinely for this purpose in detecting tin, tungsten, and molybdenum. Copper, bismuth, and lead are examples of such metals. The black copper-dithiol obscures the presence of other colored metal-dithiols, especially the bright red of stannous-dithiol (Table I). Bismuth-dithiol has a red color and its removal is necessary in the detection of tin. If the solution to be tested has been exhaustively extracted with excess dithizone, iron may remain. This ferric-dithiol, black in appearance, does not dissolve in butyl acetate. Separation is accomplished by filtration of the butyl acetate layer before the measurement is made with the photometer. The data (Table I) indicate that small amounts of the three metals, tin, iron, and copper, may be present without greatly affecting the recovery of tungsten.

Molybdenum-dithiol precipitates in the presence of citrate, while tungsten remains in solution. Citric acid is satisfactory to prevent the precipitation of tungsten, especially in the low range 0 to 50 μg. This permits the individual determination of tungsten and molybdenum when the two are present in the same solution. Molybdenum may be precipitated with dithiol from an acid solution which also contains 2% of citric acid (Table II). The molybdenum-dithiol may then be separated by solution in butyl acetate, or by filtration. The water layer, or filtrate, which contains the tungsten is evaporated and the organic matter in the residue destroyed, using nitric and sulfuric acids, in the usual manner. The tungsten may then be determined.

TABLE I.—INFLUENCE OF THE METALS, TIN, IRON AND COPPER, ON THE TUNGSTEN TEST

Test No.[a]	Metal	Photometer Reading	Remarks
1	Tin	36	Green color of precipitate obscured. Some tin-dithiol visible. Tin-dithiol dissolved completely in butyl acetate.
2	Tin	39	
3	Iron	37	Tungsten–dithiol precipitate and its solution in butyl acetate were normal in all respects.
4	Iron	37	
5	Copper	36	Dirty green precipitate. Copper-dithiol did not dissolve. The solution of tungsten-dithiol in butyl acetate appeared normal.
6	Copper	35	

[a] 100 μg. of foreign metal present in each test solution. 25 μg. of tungsten present in each test solution.

TABLE II.—INHIBITING EFFECT OF CITRIC ACID ON THE FORMATION OF TUNGSTEN-DITHIOL

Tungsten in Test Solution, μg.	Tungsten Recovered as Tungsten-Dithiol, μg.	Tungsten Not Precipitated as Tungsten-Dithiol, %
1,000	2.3	99.77
1,000	3.5	99.65
2,500	6.9	99.72
5,000	7.9	99.84
7,500	16.9	99.77
10,000	29.5	99.70
	Average	99.74

An alternative procedure may be used to estimate tungsten and molybdenum when both are present together. The reading for the mixed molybdenum-dithiol and tungsten-dithiol, dissolved in butyl acetate, is made with the photometer. For instance, two samples of exactly the same weight are assayed as described for the estimation of tungsten (see under "Methods") except that citric acid is added to one of the solutions just before the addition of the dithiol. Only the molybdenum present is responsible for the photometer reading when citric acid is present. The difference in the readings for the two samples is due to tungsten.

The methods described in this article are supported by data obtained using the Beckman Spectrophotometer, Model DU. These data, for butyl acetate solutions of tungsten-dithiol and molybdenum-dithiol, are presented graphically in Figs. 1, 2, and 3. It is necessary to remember in interpreting the data that the butyl acetate solutions presumably contain some water and some dithiol, since the reaction mixture is shaken with butyl acetate. Blanks, prepared by extracting the reaction mixture with butyl acetate, have a small difference in absorption, in comparison to the pure butyl acetate, from 550 mμ toward the ultraviolet. In the range 550 to 750 mμ, these blanks and the butyl acetate have zero absorption.

Figure 1 has three curves which are plots of the extinctions, $E = \log_{10} I_0/I$, against wave lengths. Curve *W* is for 1 μg. of tungsten per cc., dissolved as its dithiol compound in butyl acetate. Similarly, Curve *Mo*, represents 1 μg. of molybdenum per cc.; and Curve *Mo* + *W*, 1 μg. of molybdenum and 1 μg. of tungsten per cc. These curves show satisfactory maxima for photometric work.

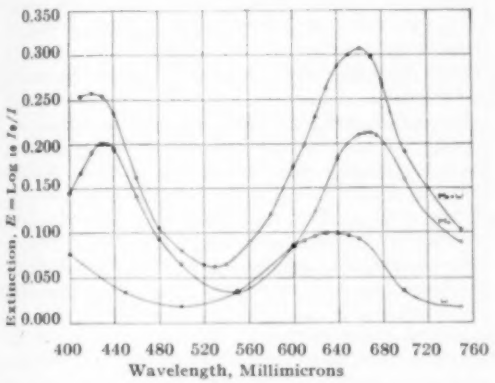


Fig. 1.—Absorption spectra of solutions of tungsten-dithiol and molybdenum-dithiol in butyl acetate.

Figure 2 has the solid line curve *Mo* of Fig. 1. The broken line curve is for molybdenum, 1 μg. per cc., separated from an equal quantity of tungsten by the use of citric acid to prevent the precipitation of tungsten-dithiol. Figure 2 is the graphic evidence of the effectiveness of citric acid for the separation of the two metals, molybdenum and tungsten, since the two curves coincide, within experimental error.

Figure 3 has curves illustrating the simple additive nature of the absorptions for tungsten-dithiol and molybdenum-dithiol, dissolved in butyl acetate. The solid line curve is the Curve *Mo*+*W* of Fig. 1. The broken line curve is the sum of the Curve *W* and the Curve *Mo* of Fig. 1. The absorption of a solution of tungsten-dithiol and molybdenum-dithiol in butyl acetate is equal to the sum of the absorptions measured individually.

The special concern of the methods described is the accurate determination, from the practical standpoint, of tungsten and molybdenum whether the two metals are present separately or together in the unknown material. It is convenient to measure the absorption of a solution containing both the tungsten-dithiol and molybdenum-dithiol, and then subtract the absorption of the solution containing

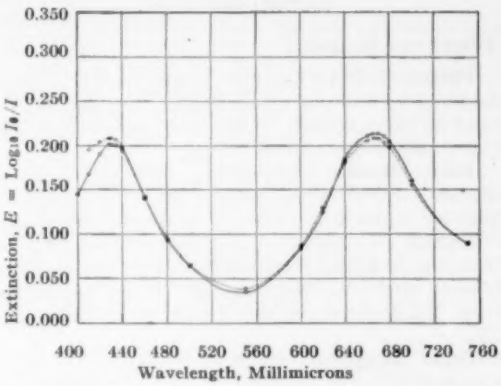


Fig. 2.—Absorption spectrum of a solution of molybdenum-dithiol, precipitated in the presence of tungsten and citric acid.

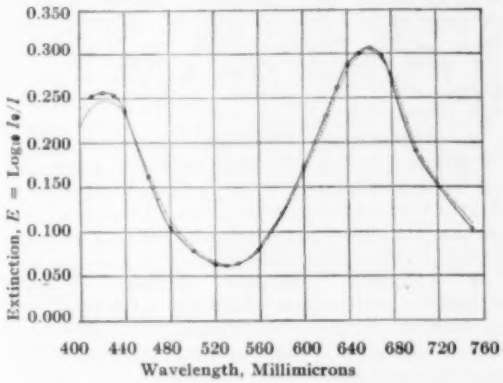


Fig. 3.—Illustration of the additive nature of the absorption spectra of tungsten-dithiol and molybdenum-dithiol.

only the molybdenum-dithiol, obtaining in this way the absorption due to the tungsten-dithiol. Actual experimental work shows that the procedure outlined is essentially correct and may be used routinely up to 25 μg. of each metal. Numerical data, the extinctions, $L = 2 - \log_{10}$ (% transmission), are given in Table III for actual solutions of tungsten-dithiol and molybdenum-dithiol in butyl acetate. These data show also the adequacy of the described methods for the estimations of tungsten and molybdenum when both are present together.

TABLE III.—EXTINCTIONS FOR BUTYL ACETATE SOLUTIONS OF TUNGSTEN-DITHIOL AND MOLYBDENUM-DITHIOL

(Filter Range, 610 to 660 mμ. Extinction, $L = 2 - \log_{10}$ (% transmission). Volume = 25 cc.)

Tungsten and Molybdenum Separately			Tungsten and Molybdenum in the Presence of Each Other		
μg. in Solution	Extinctions		μg. in Solution		Extinctions
	W	*Mo*	*W*	*Mo*	
5	0.10	0.13	5	5	0.23
10	0.19	0.25	10	10	0.45
15	0.28	0.37	15	15	0.68
20	0.38	0.51	20	20	0.91
25	0.47	0.64	25	25	1.13
50	0.96	1.21	..	..	..

EXPERIMENTAL

Filters and Reagents

Filters.—Filters No. 978½ and No. 244, manufactured by Corning Glass Works, Corning, N. Y., used in combination. Optimum transmission for this combination, 610 to 660 mμ.

Butyl Acetate.—Redistilled commercial butyl acetate. Butyl acetate used in the tests is satisfactory for re-use after recovery by simple distillation.

Dithiol.—4 - Methyl - 1,2 - dimercaptobenzene in sealed glass ampuls, prepared by The British Drug Houses, Ltd., London, England. When impossible to obtain elsewhere, dithiol is made in the laboratory (9).

Other Reagents.—U. S. P. XIII or Reagent Grade.

Preparation of Solutions Used in Tests

1. Ammonium Sulfate-Phosphoric Acid Solution, So-called "Salt Solution."—Dissolve 600 Gm. of ammonium sulfate in 1500 cc. of water. Add 80 cc. of 85% phosphoric acid, and adjust to *p*H 1.8 with ammonia. Dilute to 2000 cc. Filter before use. This solution contains about 0.3 Gm. ammonium sulfate in 1 cc.

2. Dithiol Reagent Solution.—Add 0.1 to 0.2 Gm. of dithiol(4-methyl-1,2-dimercaptobenzene) to 100 cc. of approximately 0.25 *N* sodium hydroxide. Add 0.5 cc. of thioglycolic acid. This solution is prepared in sufficient quantity for one day's use and is kept in the icebox when not actually in use.

3. Tungsten Standard Solution.—Dissolve 0.0449 Gm. of sodium tungstate ($Na_2WO_4{\cdot}2H_2O$) in 1000 cc. of water containing 10 cc. of concentrated ammonia. This solution contains 25 μg. of tungsten in 1 cc.

4. Molybdenum Standard Solution.—The quantity of molybdic acid taken must correspond to its purity. For example, dissolve 0.0442 Gm. of molybdic acid, 85% pure MoO_3, in 1000 cc. of water containing 10 cc. of concentrated ammonia. This solution contains 25 μg. of molybdenum in 1 cc.

5. Dilute Dithizone Solution.—Dissolve about 0.01 Gm. of diphenylthiocarbazone (dithizone) in 1000 cc. of redistilled chloroform.

6. Strong Dithizone Solution.—Dissolve about 0.10 Gm. of dithizone in 1000 cc. of redistilled chloroform.

Distilled Water.—In order to avoid metal contamination, distilled water for this work is specially prepared using an all-Pyrex glass apparatus.

METHODS

Determination of Tungsten (General)

Procedure: Destruction of Organic Matter.—Clean several 30-cc. Pyrex Kjeldahl flasks with hot dilute hydrochloric acid (1 + 1). Rinse with distilled water, then rinse with ammonia, finally removing traces of the ammonia by thoroughly rinsing with distilled water. Weigh sample into a 30-cc. Kjeldahl flask using sufficient sample (representing about 25 μg. of tungsten, which is an optimum quantity of tungsten for the test). Add 4 cc. of sulfuric acid (1 + 3). Digest sample with concentrated nitric acid until the destruction of organic matter is complete, adding the nitric acid portionwise, and in small amounts. Cool to room temperature by removing the source of heat. Add about 3 cc. of water. Again cool contents in an ice-water bath. Add 5 cc. of ammonium hydroxide and evaporate until salts crystallize out. Gently fuse salts. Cool to room temperature by removing the source of heat and add a few cc. of water to dissolve the fused salts. Cool further in an ice-water bath and add 5 cc. of ammonium hydroxide. Evaporate the solution and fuse the salts. Cool to room temperature by removing the source of heat. Add about 3 cc. of concentrated nitric acid and boil gently until essentially all the nitric acid has been removed. This is manifested when the liquid residue in the flask just ceases to effervesce. Cool to room temperature by removing the source of heat.

Adjustment of Ammonium Sulfate Concentration, *p*H, and Precipitation.—Enough Ammonium Sulfate-Phosphoric Acid Solution is added so that 4 Gm. of ammonium sulfate in 20 cc. is present. Since 4 cc. of sulfuric acid (1 + 3) is used, this will probably be about 8 cc. of the solution. Boil and cool the solution. Adjust to a *p*H of 1.5 to 1.8 with ammonium hydroxide using a glass electrode. Adjust volume to 18 cc. Add 2 cc. of Dithiol Reagent Solution. Place flask in boiling water bath for thirty minutes. If a large amount of precipitate is present, add an additional 2 cc. of Dithiol Reagent Solution. Chill contents in an ice-water bath.

Extraction and Measurement.—Extract the tungsten-dithiol with a 15-cc. portion of butyl acetate. Repeat extraction with an additional 10-cc. portion of butyl acetate. Filter the butyl acetate solution of the extracted tungsten-dithiol through filter paper (No. 4 Whatman). Add additional solvent, if necessary, to make the total volume equal 25 cc. Read the intensity of color with photometer using a suitable red filter. The tungsten present in the unknown is calculated by comparing with 25 μg. of tungsten in solution, as tungsten-dithiol, which is prepared as follows: Add 1 cc. of Tungsten Standard Solution to 13 cc. of "Salt Solution" contained in a 30-cc. Kjeldahl flask. Adjust volume, *p*H, etc., as above under "Adjustment of Ammonium Sulfate Concentration, *p*H, and Precipitation." The 25-μg. standard in 25 cc. of butyl acetate, as tungsten-dithiol, may be diluted for comparison as needed. A faulty adjustment of the *p*H just prior to adding the Dithiol Reagent Solution may occur, especially if many determinations are being made at the same time. Although such a faulty adjustment of the *p*H happens rarely, it is necessary to determine the *p*H of the water layer after the extraction, with butyl acetate to remove the tungsten-dithiol, in order to be certain that the *p*H is within the limits 1.5 to 2.5. A new determination of tungsten should be made, starting with a new sample of the unknown, if the *p*H of the water layer is outside the range 1.5 to 2.5.

Modified Method, When Tungsten Is Present in Very Small Amounts, 0 to 10 μg.—A modification of the above general method may be employed when the amount of tungsten present is less than 10 μg.

Procedure.—Digest the sample in the usual manner to destroy organic matter, omitting fusions. Cool to room temperature by removing source of heat. Dilute to 5 cc. with water and add 1 cc. of 10% citric acid solution. Boil to expel fumes and cool in an

ice-water bath to room temperature. Transfer to a small separatory funnel. Add 1 cc. of the Dilute Dithizone Solution. Add ammonia dropwise, shaking contents of funnel until an intense red color appears in the chloroform layer. Completely remove the dithizone metals using the Strong Dithizone Solution. When all the metal dithizonates have been removed, the water layer is a deep brownish yellow in color due to an excess of dithizone. Add 10 cc. of chloroform to the contents of the separatory funnel and shake the mixture. The chloroform layer must be bright green in color. Discard all chloroform layers. Transfer water layer to a small test tube, evaporating the water portionwise. Rinse the separatory funnel with a few cc. of distilled water, and transfer the rinsing to the small test tube. Add sulfuric acid (1 + 3) in an amount sufficient to make a final ammonium sulfate concentration of 1 Gm. in 5 cc. Redigest sample with nitric acid until the destruction of organic matter is complete. Cool to room temperature by removing the source of heat. Add 1 cc. of water to the test tube, and cool in an ice-water bath. Add 2 cc. of concentrated ammonium hydroxide. Evaporate the water and gently fuse the contents of the test tube. Cool again to room temperature by removing the source of heat. Add 1 cc. of water to dissolve the fused salts. Evaporate the solution and again fuse the salts. Cool to room temperature by removing the source of heat. Add 1 cc. of water to dissolve the fused salts. Add 0.25 cc. of sulfuric acid (1 + 3). Add 3 drops of 85% phosphoric acid. Heat the solution to boiling and cool to room temperature in an ice-water bath. Add 1 cc. of Dithiol Reagent Solution and adjust to a *p*H of 1.8 with ammonia using a glass electrode. Dilute to 5 cc. with water. Heat sample in a boiling-water bath for thirty minutes. Cool in an ice-water bath. Compare, visually, the blue-green tungsten-dithiol precipitate with standards prepared in a similar manner (0, $^1/_2$, 1, $1^1/_2$, 2, $2^1/_2$, 3, 4, 5, and 10 μg. of tungsten).

Determination of Molybdenum (General)

Molybdenum may also be determined by proceeding exactly as outlined under "Determination of Tungsten (General)," except that it is necessary to add 0.5 Gm. of citric acid just prior to the final adjustment of *p*H and volume.

Modification A (Rapid).—The molybdenum-dithiol precipitates readily in dilute sulfuric acid solution, and it is not necessary to adjust the *p*H carefully. The general procedure may be modified by omitting, in the case of molybdenum, the careful adjustment of *p*H, using instead a dilute sulfuric acid solution.

Procedure.—Prepare sample as described under "Determination of Tungsten (General), Destruction of Organic Matter." Add to the cold solution 4 cc. of sulfuric acid (1 + 3) and 3 drops of 85% phosphoric acid. Add 0.5 Gm. of citric acid. Dilute with water to 20 cc. and add 2 cc. of Dithiol Reagent Solution. Allow the mixture to stand at room temperature for about two hours. Extract with butyl acetate, and determine color intensity as described under "Determination of Tungsten (General), Extraction and Measurement." Twenty-five micrograms of molybdenum, 1 cc. of the Molybdenum Standard Solution, precipitated and extracted as described in this paragraph, serves as a standard. If tungsten is to be determined after the estimation of molybdenum, then the water layer is evaporated, and the residue treated to destroy organic matter in the usual manner. Tungsten is then determined as given under "Determination of Tungsten (General)."

Modification B (Rapid).—Considerable time may be saved if it is known from experience that the color transferred to the butyl acetate layer is due only to the molybdenum-dithiol formed and that the organic matter present does not interfere with the complete precipitation of the molybdenum-dithiol.

Procedure.—Transfer an accurately weighed sample, representing about 25 μg. of molybdenum, to a 25-cc. glass-stoppered graduated cylinder. Add 4 cc. of sulfuric acid (1 + 3) and 3 drops of 85% phosphoric acid. Add 0.5 Gm. of citric acid. Add 10 cc. of water and mix. Add 2 cc. of Dithiol Reagent Solution. Dilute with water to a volume of 25 cc., mix, and allow to stand at room temperature for about two hours. Extract with butyl acetate and determine color intensity as described under "Determination of Tungsten (General), Extraction and Measurement." Twenty-five micrograms of molybdenum, 1 cc. of the Molybdenum Standard Solution, precipitated and extracted as described in this paragraph, serves as a standard.

Determination of Tungsten and Molybdenum When Both Are Present Together in the Sample

Method I.—The molybdenum-dithiol formed is removed before the determination of tungsten is made.

Procedure.—Determine molybdenum as described under "Determination of Molybdenum (General)," using "Modification A" or "B." Retain the water layer from the butyl acetate extraction. Evaporate this water layer and digest in the usual manner to destroy organic matter. Determine tungsten as described under "Determination of Tungsten (General)," making due allowance for the amount of sulfuric acid present from the molybdenum test.

Method II.—The molybdenum-dithiol is not removed by extraction with butyl acetate before the determination of tungsten is made.

Procedure.—Proceed exactly as described under "Determination of Tungsten (General)" starting with two samples of exactly the same weight. Add 0.5 Gm. of citric acid to one of the samples just prior to final adjustment of *p*H and volume. Heat in a boiling water bath for thirty minutes. Prepare both a tungsten standard and a molybdenum standard, adding 0.5 Gm. of citric acid to the molybdenum standard. The extinction of the tungsten may be found by subtracting the extinction of the sample containing citric acid from the extinction of the sample containing no citric acid. The amount of tungsten or molybdenum may then be calculated by reference to the proper standard.

DISCUSSION AND SUMMARY

General methods are described for tungsten and molybdenum using the reagent "dithiol" (4-methyl-1,2-dimercaptobenzene). Dithiol may be considered a specific reagent for tungsten and molybdenum, because the common metals apparently do not form

metal-dithiols which dissolve in butyl acetate and impart to this solvent a stable characteristic color. In general, there is associated with a specific metal-dithiol a characteristic color which partly identifies the metal present. An example is the bright red precipitate of stannous-dithiol. Similarly, tungsten and molybdenum yield highly colored precipitates with "dithiol."

Spectrophotometric data for butyl acetate solutions of tungsten-dithiol and molybdenum-dithiol are presented which support the methods described.

This reagent precipitates tungsten-dithiol, an insoluble, blue-green compound, from an aqueous solution, *p*H 2. Butyl acetate dissolves the tungsten-dithiol, yielding a solution, blue-green in color, which is suitable for photometric measurement. The method is extremely sensitive, readily detecting as little as 0.2 μg. of tungsten, dissolved as tungsten-dithiol, in 1 cc. of butyl acetate. Moreover, the determinations of tungsten made according to the method are very satisfactory, consistent and reproducible values being obtained. The method is not difficult. No tedious separations are involved, since small amounts of the metals, such as iron, copper, and tin, cause little interference.

Although the general method describes the solution of tungsten-dithiol in butyl acetate and the photometric measurement of the color intensity of butyl acetate solution, it is not necessary to carry out these steps if only very small amounts of tungsten are present. A good gradation in the amounts of the blue-green tungsten-dithiol precipitate is noted for changes of 1 μg. of tungsten when quantities up to about 10 μg. are present. A modification of the general method is given for amounts of the tungsten less than 10 μg. The simple visual estimation of the amounts of the tungsten-dithiol precipitate is substituted for the longer photometric estimation. If a series containing known amounts of tungsten as tungsten-dithiol precipitate, is prepared in approximately 1 μg. steps, the amount of tungsten in the unknown may be readily determined by simple visual comparison.

The general method is applicable for the estimation of molybdenum without modification if tungsten is absent, since the molybdenum-dithiol precipitates readily in an aqueous solution, the *p*H of which is less than 2.5. Molybdenum-dithiol, like tungsten-dithiol, dissolves easily in butyl acetate, yielding a solution, yellow-green in color, which is suitable for photometric measurement. The addition of citric acid prevents the precipitation of tungsten-dithiol, but apparently does not interfere with the precipitation of molybdenum-dithiol. The general method for tungsten is changed to a general method for molybdenum by simply adding citric acid to the test solution.

Small amounts of molybdenum, under 10 μg., may be estimated as described for similar quantities of tungsten. If a series containing known amounts of molybdenum, as molybdenum-dithiol precipitate, is prepared in approximately 1 μg. steps, the amount of molybdenum in the unknown may be readily determined by simple visual comparison.

A more precise control of the experimental conditions appears necessary during the precipitation of tungsten-dithiol than during the precipitation of molybdenum-dith ol. Three factors which influence the tungsten test considerably are: (*a*) the *p*H of the test solution; (*b*) the heating of the test solution; (*c*) the presence of citric acid in the test solution. The *p*H of the test solution should be kept within the limits, 1.5 to 2.5. Heating the test solution facilitates the precipitation of tungsten-dithiol. Citric acid prevents the precipitation of tungsten-dithiol. In contrast, the above three factors appear to have little influence on the precipitation of the molybdenum-dithiol, since this compound separates rapidly in dilute sulfuric acid solution at room temperature and in the presence of citric acid. This behavior of molybdenum enables time-saving modifications of the general method to be made. A modification omitting the precise adjustment of *p*H and using instead a dilute sulfuric acid solution is described. A second modification of the general method may be used when it is known that no interference results from the organic matter present. In this second modification, both the destruction of the organic matter and the precise adjustment of *p*H are omitted. The heating of the test solutions is omitted in both modifications.

It is also possible to separate the molybdenum rapidly from a test solution containing both molybdenum and tungsten, using the described modifications of the general method for the determination of molybdenum. The tungsten remains in the water layer. After evaporation of the water layer, the tungsten is determined following exactly the described general method for tungsten.

When both molybdenum and tungsten are present together in the sample material, the general methods for tungsten and molybdenum are modified in order that the two metals may be determined readily without actual separation. Starting with two test samples of exactly the same weight, citric acid is used to prevent the formation of tungsten-dithiol in one of the sample solutions. The color intensity of the dissolved dithiol precipitates is read for each sample solution, using the same photometer arrangement. The difference in the readings of the two solutions is due to tungsten.

Dark-colored precipitates are found with certain samples at the interface of the butyl acetate and water layers. When an undesirable metal-dithiol is present, the butyl acetate and water layers should be shaken in the separatory funnel during a period of several minutes before removing the butyl acetate layer.

The step (see under "Determination of Tungsten (General), Destruction of Organic Matter"), boiling the digested residue in a Kjeldahl flask with a final quantity of nitric acid and then removing essentially all the nitric acid, is very important. Apparently all the tungsten in the test solution is converted to compounds of tungsten from which the precipitation as tungsten-dithiol is complete. Nitric acid and ammonium nitrate, when present in very small amounts, do not adversely affect the test.

Experience has indicated that the test solution should contain an excess of sulfuric acid, before adding the ammonium sulfate solution and adjusting the *p*H. If the solution is strongly ammoniacal and phosphoric acid is used for the adjustment of the *p*H, unsatisfactory results are obtained; approximately one-half of the tungsten content being found.

Relatively large amounts of manganese, ferrous, and ferric ions may be present in the test solution. Actually the presence of these ions is not desirable;

however, if they are not present in excessive amounts, no apparent interference results. If the solution contains a relatively large amount of copper, the copper is removed with dithizone before completing the test.

Occasionally a small amount of a precipitate, light green in color, may be observed suspended in the butyl acetate layer which is used to dissolve the tungsten-dithiol. The precipitate collects on the filter paper during the filtration of the butyl acetate layer. The determination of tungsten should be repeated if a green precipitate is observed on the filter paper, because this precipitate is apparently a tungsten compound.

Molybdenum appears to form a more stable compound than does tungsten. Also, the molybdenum compound forms much more readily and is not greatly affected by moderately high concentrations of acids. However, the *p*H of 2.5 should not be exceeded since high *p*H values adversely affect the test.

CONCLUSIONS

Detailed methods and also modifications of such methods are described to estimate tungsten and molybdenum using the reagent dithiol. Dithiol is perhaps the most satisfactory reagent to use routinely for the colorimetric estimation of tungsten and molybdenum in the range 0 to 50 micrograms. The estimation of tungsten and molybdenum in the presence of each other is described. Citric acid prevents the precipitation of tungsten with dithiol sufficiently to permit a satisfactory determination of molybdenum.

Spectrophotometric data, which support the described methods, are presented.

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Yellow Phenolphthalein*

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The chemistry and pharmacology of yellow phenolphthalein are reviewed. The method of isolation and partial characterization of a compound not heretofore reported are described. Methods for the determination of toxicity and laxative activity of yellow phenolphthalein are given. The data recorded indicate that yellow phenolphthalein is two and one-half times more active than phenolphthalein of U. S. P. quality.

PHENOLPHTHALEIN is probably the most frequently used synthetic organic laxative. Because it is odorless, tasteless, and stable, it lends itself readily to incorporation in pleasant-tasting vehicles and mineral oil emulsions. These properties have prompted its use in various preparations for the relief and treatment of constipation.

HISTORICAL

Baeyer (1) was the first to prepare this compound in 1871, and gave it the name phenolphthalein because of its derivation from phenol and phthalic acid. He also mentioned a light yellow phenolphthalein which he obtained in one of his experiments (2).

The laxative action of phenolphthalein was discovered by Vamossy (3) in 1900; its therapeutic value was quickly recognized and it was included in the pharmacopœias of almost every country where an official classification of drugs was made.

In the process of production of phenolphthalein, a stage is reached where certain by-products formed in the synthesis have not yet been removed (4). There are yellow to brownish yellow bodies which at this stage impart to the product its characteristic color, hence the name yellow phenolphthalein. The intensity of the color is determined by the quantity of yellow bodies present. Further processing removes these colored bodies, resulting in a substance known as white phenolphthalein. This is the U. S. P. product.

Williams, Abramowitz, and Killian (5) found yellow phenolphthalein 3.3 times more active, and Loewe and Hubacher (6) 2.9 times more active, than the white variety. Recent pharmacological determinations in this laboratory, using rhesus monkeys and employing an improved technique showed yellow phenolphthalein on the average 2.5 times more active as a laxative than the U. S. P. product.

Wohlmuth (7), who was the first to describe the

* Received November 24, 1947, from the Research Laboratory of Ex-Lax, Inc., Brooklyn, N. Y.
† The authors are indebted to Dr. A. A. White, Jr., for editorial assistance.

yellow-colored phenolphthalein in the medical literature, found it nonpoisonous. Vamossy considered it a harmless laxative (3*b*). Fantus and Dyniewicz (8) "found it impossible to kill animals—daphnia, worms, mice, cats, dogs, pigs, monkeys—with phenolphthalein administered in any dose." In our laboratory, rhesus monkeys, receiving in their food 100 times the individual threshold dose of yellow phenolphthalein established for the particular animal, showed no ill effects.

The use of yellow phenolphthalein has become increasingly greater because its laxative action is approximately 2.5 times that of phenolphthalein U. S. P., without exhibiting any other pharmacologic or toxicologic change corresponding to the increased physiological action. Since the laxative action of phenolphthalein is reduced by further processing, without any therapeutic benefit accruing from it, it would appear to be an unproductive step, hardly justified economically by the result obtained, that of merely changing the color from yellow to white.

Since there is little published information available concerning the chemical and pharmacologic properties of yellow phenolphthalein, an investigation was undertaken to learn more about this drug, and perhaps discover the reason for its greater laxative activity.

CHEMICAL

It was thought that the yellow-colored substance or substances present in yellow phenolphthalein might be formed from traces of unknown by-products contained in the raw materials used in the manufacture. However, since phenol and phthalic anhydride from different sources and of highest purity always produced yellow phenolphthalein, this was considered unlikely. The yellow-colored compound evidently is formed from phenol and phthalic anhydride.

An attempt was made to find out whether hydroxyanthraquinones are present in yellow phenolphthalein. In several published reports (9) it is suggested that such substances are present, although their presence has never been proved. Two considerations may explain why hydroxyanthraquinones became associated with yellow phenolphthalein:

1. The two monohydroxyanthraquinones are yellow compounds and are obtained in small yields on heating phenol with phthalic anhydride at higher temperatures in the presence of sulfuric acid (2, 10). Because phenolphthalein is manufactured from these same raw materials, but at a lower temperature, it was considered likely that the same yellow hydroxyanthraquinones might form in small amounts, together with the phenolphthalein.

2. From a number of plant laxatives, such as rhubarb, senna, cascara, and aloe, yellow compounds were isolated. These were identified as polyhydroxyanthraquinones, present in the plant as glycosides. The laxative properties of these plant laxatives are attributed, partly at least, to these hydroxyanthraquinones. It was erroneously reasoned that, since the compounds which produce the yellow color in yellow phenolphthalein are hydroxyanthraquinones and since some of these compounds are laxative, the increased laxative action of yellow phenolphthalein is due to the presence of these compounds.

A search was made for hydroxyanthraquinones in yellow phenolphthalein, especially for the 1- and 2-hydroxyanthraquinones, which are the ones that can form from phenol and phthalic anhydride. A quantitative method for the determination of hydroxyanthraquinones was developed, based on their property to sublime. Not a trace of hydroxyanthraquinones could be detected. Spectroscopic examination failed to reveal the presence of compounds of the class of hydroxyanthraquinones.[1] Furthermore, the laxative properties of hydroxyanthraquinones alone, or mixed with phenolphthalein U. S. P., were less pronounced than that of phenol phthalein U. S. P. alone. The greater laxative activity of yellow phenolphthalein, therefore, cannot be ascribed to the presence of hydroxyanthraquinones.

A systematic attempt to isolate the compounds present in yellow phenolphthalein was then begun. The separation of several compounds was accomplished by fractional crystallization, followed by chromatographic adsorption methods or molecular distillation. The following compounds, all of which are colorless, have been isolated up to the present time: phenolphthalein (I), fluoran (II), isophenolphthalein or 3-(*o*-hydroxyphenyl)-3-(*p*-hydroxyphenyl)phthalide (III), phenolphthalin (IV), a compound melting at 250° (V) and one melting at 235° (V*a*), as well as traces of 2-(4-hydroxybenzoyl)-benzoic acid (VI).

The presence of these compounds can be easily explained, because they form from phenol and phthalic anhydride. Fluoran has always been known to form in small quantities in the synthesis of phenolphthalein. Isophenolphthalein, an isomer of phenolphthalein, has one of the two OH groups in the *ortho* position to the central carbon atom, and the other in *para* position. It was first discovered by Orndorff and Barrett (11) in the residue left from the manufacture of phenolphthalein.

Since isophenolphthalein was found in all the yellow phenolphthaleins analyzed, it can now be stated with certainty that this phthalein is formed regularly in small quantities along with fluoran, in the production of phenolphthalein. It is because of its close physical and chemical resemblance to phenolphthalein that isophenolphthalein was not previously discovered in yellow phenolphthalein. Chemical textbooks, which usually mention the formation of fluoran in connection with the preparation of phenolphthalein, may well mention that isophenolphthalein is also formed in the manufacture from phenol and phthalic anhydride. Phenolphthalin, a reduction product of phenolphthalein (12) was found in only one sample of yellow phenolphthalein.

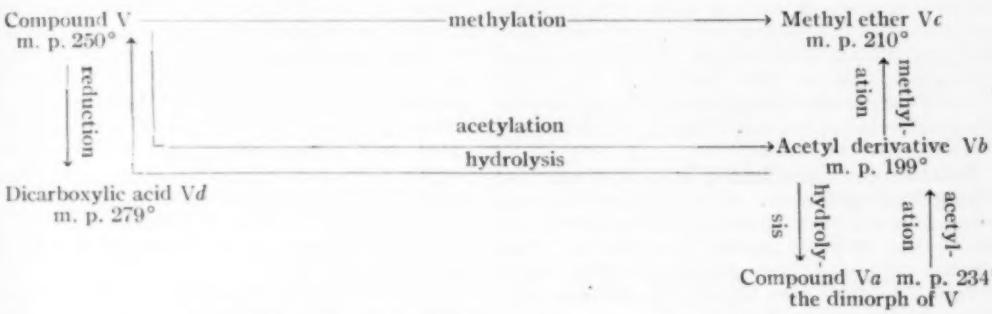
The acid, 2-(4-hydroxybenzoyl)-benzoic acid, is assumed to be an intermediate in the formation of phenolphthalein (13), but it also forms by the air oxidation of dilute alkaline solutions of phenolphthalein (14).

Compound V, melting at 250°, has the probable empirical formula of $C_{34}H_{20}O_6$, which suggests its formation from 2 moles of phthalic anhydride and 3 moles of phenol, with the elimination of 3 moles of water. Formulas $C_{34}H_{22}O_6$ or $C_{35}H_{22}O_6$ must also be considered for this compound.

[1] The spectroscopic examination was made by E. F. Williams in 1934 and by R. H. Muller, of New York University, in 1938.

Compound V has a molecular weight of about 524. That compounds of such high molecular weight can form in the preparation of phthaleins was recently discovered in this laboratory. The results of these experiments, when completed, will be published.

Compound V dissolves but slowly in 2.5 *N* sodium hydroxide, the behavior indicating a lactone. The alkaline solution is colorless, but turns pink on heating. On acidification of this solution, a gelatinous precipitate forms, from which the original compound V, and also a new compound V*a*, melting at 234°, can be isolated. The latter compound is a dimorph of compound V, because it contains the same percentage of carbon and hydrogen, and a mixture of the two causes no depression of the melting point. On acetylation of compound V or V*a*, the same acetyl derivative (V*b*) is obtained. Compound V forms a monoacetyl derivative (V*b*) and a monomethyl ether (V*c*). On reduction, a dicarboxylic acid (V*d*) is obtained, which in turn can be decarboxylated. These data show that compound V has one OH group and two lactone rings. The lactone rings are probably similar to the one present in phthaleins. The tentative formula for compound V, therefore, can be assumed to be $C_{22}H_{10}O\left(\begin{matrix}C{=}O\\ \diagdown\\ -O\end{matrix}\right)_2(OH)$. The interrelation of the derivatives of compound V are shown in the following scheme:



There is an indication of the presence in yellow phenolphthalein of one or more phthaleins other than phenolphthalein and isophenolphthalein. This is assumed from the observation that preparations of yellow phenolphthalein, from which the bulk of phenolphthalein has been removed by fractional crystallization, form red, nonfading solutions in dilute sodium hydroxide. Solutions of phenolphthalein and isophenolphthalein of the same concentration, and kept under the same conditions, gradually fade (14). Furthermore, traces of other compounds were isolated.

This amorphous brown residue,[2] remaining after most of the phenolphthalein has been removed from yellow phenolphthalein by fractional crystallization in the process of manufacture of phenolphthalein, was also investigated. The same compounds were isolated as were found in yellow phenolphthalein.

PHARMACOLOGICAL

With one exception, all the compounds which had been separated from yellow phenolphthalein were tested for their laxative action on the rhesus monkey (Table II). Compound V was not tested, the entire quantity isolated being consumed in the chemical tests. Neither fluoran nor phenolphthalin was found to possess perceptible laxative action, corroborating the previous findings of Loewe and Hubacher (6). These workers found that isophenolphthalein was only slightly less active than phenolphthalein, while Hatcher, in a publication of Orndorff and Barrett (11), also Blicke and Weinkauff (9), reported it to be inactive. In order to clear up this difference in observations, the physiologic action of isophenolphthalein was carefully redetermined. Repeated tests showed that isophenolphthalein is inactive in monkeys as well as in humans.

The two monohydroxyanthraquinones were found to have practically no laxative action. Of the two dihydroxyanthraquinones tested, the 1,8-derivative was about one-half as active as phenolphthalein, the result being in agreement with potency figures published (15). This 1,8-dihydroxyanthraquinone, known also as chrysazin, was for a time used as a laxative for human use, under the names of istizin or istin (15) and is still used for such purpose in veterinary medicine under the name danthron (16).

Mixtures of hydroxyanthraquinones with phenolphthalein did not increase the laxative action of the combination, indicating that they are not activators of phenolphthalein.

The presence of still other compounds in yellow phenolphthalein is clearly suggested by the colored, resinous substances remaining. Further investigations are now under way with these residues, and the result may shed light on the subject.

EXPERIMENTAL[3]

Physical and Chemical Properties of Yellow Phenolphthalein.—The commercially available yellow phenolphthalein, manufactured during the years 1938–1946 by Monsanto Chemical Company or by Charles Pfizer and Company, is a powder of moderate yellow color.[4] It melts at 255–260°, has a specific gravity 20°/4° = 1.290–1.296. It contains up to 0.3% fluoran, and not more than 0.1% ash. Yellow phenolphthalein has practically the same

[2] This residue was obtained through the courtesy of Monsanto Chemical Company, St. Louis, Mo., and Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

[3] All melting points are corrected. The carbon and hydrogen figures are the average of 3 to 8 analyses. Elementary analyses were made by Arlington Laboratories, William Saschek, and Carl Tiedke.

[4] This is the designation for this color in the Munsell Color System "Methods of Designating Colors," Research Paper RP 1239, Bureau of Standards, Washington, September, 1939.

solubility as phenolphthalein U. S. P. XIII (17). One gram dissolves in 12 cc. alcohol, and in 102 cc. ether U. S. P. XII at 25°. The alcoholic solution (1 Gm. in 15 cc.) is moderate yellowish orange,[4] and some of these solutions show a slight greenish fluorescence in reflected light. Yellow phenolphthalein does not contain any sulfur, and analyzes 75.20 ± 0.3% C and 4.39 ± 0.11% H. (Theory for phenolphthalein $C_{20}H_{14}O_4$: C, 75.46%; H, 4.47%.)

Test for Hydroxyanthraquinones in Yellow Phenolphthalein.—Mixtures of hydroxyanthraquinones with phenolphthalein U. S. P. were prepared, either by mixing in a mortar or by coprecipitation from their alkaline solution. Quantities of 1 Gm. of this mixture were subjected to sublimation at 150° and 10–20 μ pressure in Tube C of the apparatus described elsewhere (18). The phenolphthalein does not sublime to any extent under these conditions, but the two monohydroxyanthraquinones and the polyhydroxyanthraquinones do sublime. The combined yellow sublimate, obtained from 10 Gm. of the mixture, was resublimed. By this technique it was possible to obtain sufficient hydroxyanthraquinones from 10 Gm. of the mixture to make positive identification by means of the mixed melting point and by preparing their acetyl derivative. By the same method, the 1,2-, 1,4-, 1,5-, and 1,8-dihydroxyanthraquinones, as well as the above mono derivatives, could be positively identified when present in a quantity of 0.1% or more. Phenolphthalein containing 0.01% of any of the above-mentioned hydroxyanthraquinones yielded a yellow sublimate within one hour at 150° and 10–20 μ pressure. None of the different lots of yellow phenolphthalein, when subjected to the same process, yielded even a trace of yellow sublimate.

Isolation and Identification of Compounds from Yellow Phenolphthalein.—Certain precautions were observed in this work. Solutions were always heated on a water bath to prevent overheating. Since alkaline solutions of phenolphthalein slowly absorb oxygen (14), they were kept under nitrogen when exposed to the air.

The general procedure was to recrystallize 2 Kg. of yellow phenolphthalein from ethanol until the resulting crystals met U. S. P. specifications. The yellowish brown, alcoholic mother liquors were united and evaporated to dryness. The colored residue was dissolved in 0.1 *N* sodium hydroxide (1 Gm. in 80 cc.) and filtered. The insoluble part was crude fluoran, further purified by recrystallization from ethanol and sublimation *in vacuo* (18). The red filtrate was slowly run into an excess of 0.1 *N* sulfuric acid at room temperature. The dried precipitate was crystallized from acetic acid (1 Gm. in 6 cc.). The first yield was rich in phenolphthalein, which was obtained in a pure state by recrystallization from ethanol. When no more crystals could be obtained by partial evaporation of the acetic acid filtrate, the solvent was evaporated in a partial vacuum. The brownish residue resulting was dissolved in pure ether (1 Gm. in 200 cc.). The part insoluble in ether consisted principally of phenolphthalein. The ethereal yellow filtrate was now chromatographed. For the column, a tube of 50 mm. inside diameter and 600 mm. length was used. This was filled either with activated aluminum hydroxide (ALORCO, grade A), or magnesium carbonate (U. S. P. XI, "heavy powder" of the J. T. Baker Chemical Company). The various colored zones were cut out, and each one eluted several times with hot ethanol. Pure compounds were isolated from the extracts by fractional crystallization from suitable solvents, such as acetic acid or ethanol. In other instances, the extract was directly acetylated, and the compounds were isolated in the form of their crystalline acetyl derivatives. The ether which had passed through the chromatographic column contained a small amount of fluoran.

Later, it was found that partial separation of the brownish residue could be accomplished with less effort by molecular distillation in a pot-type still.[5] The fractions presented brownish masses, from which crystals could be obtained by fractional crystallization. The lower fractions contained fluoran; the middle fractions were rich in isophenolphthalein, and the last fractions contained phenolphthalein and the compound designated V. The residues in the still dissolved in 0.1 *N* sodium hydroxide with a red color, which did not fade on prolonged exposure to air.

Fluoran (II) was identified by its melting point of 183–184°, its characteristic greenish fluorescence when dissolved in concentrated sulfuric acid, and the preparation of hydrofluoronic acid, which melted at 228–231° (19). The isophenolphthalein (III) was compared by means of the mixed melting point method with a synthetic sample (m. p. 205–206°) prepared according to the method described by Blicke and Patelski (20). The diacetyl derivative of isophenolphthalein melted at 168–169°. Phenolphthalin (IV), m. p. 236–238°, was identified by comparison with an authentic sample. The 2-(4-hydroxybenzoyl)-benzoic acid (VI), melting at 211–214° with decomposition, was identified by the mixed melting point method, using a sample prepared by the method of Ullmann and Schmidt (21) and also by the process of Friedlaender (22). The diacetyl derivative of this compound has a melting point of 157.5–159.2°.

Anal.—Calcd. for $C_{18}H_{14}O_6$: C, 66.26; H, 4.29. Found: C, 66.56; H, 4.45.

Compound V crystallizes from acetic acid in the form of fine, colorless needles, arranged in tufts. It melts at 249.5–250.7° and dissolves in concentrated sulfuric acid, producing a light yellow color, which remains unchanged on heating.

Anal.—Calcd. for $C_{34}H_{20}O_6$: C, 77.85; H, 3.84; mol. wt. 524. Calcd. for $C_{34}H_{22}O_6$: C, 77.54; H, 4.21; mol. wt. 526. Calcd. for $C_{35}H_{22}O_6$: C, 78.06; H, 4.12; mol. wt. 538. Found: C, 78.02 ± 0.30; H, 4.12 ± 0.35; mol. wt. 504 ± 39,[6] 488 ± 35.[7]

At room temperature, compound V dissolves but slowly in 2.5 *N* sodium hydroxide, forming a colorless solution, which turns red on heating, assuming the same reddish magenta color[4] as that of an alkaline solution of phenolphthalein. On acidification, a gelatinous precipitate forms. On fractional crystallization from acetic acid, this precipitate yields the original compound V, and also a new compound V*a*, described later in greater detail.

[5] This distillation was performed by E. S. Perry, of Distillation Products, Inc., Rochester, N. Y.

[6] Rast method; camphor as solvent.

[7] Signer method, as described by E. P. Clark, *Ind. Eng. Chem., Anal. Ed.*, **13**, 840(1941). Acetone was used as a solvent, and azobenzene as a standard.

The acetyl derivative of compound V (V*b*) was prepared by heating a mixture of 0.20 Gm. of V, 0.50 cc. of glacial acetic acid, 0.90 cc. of acetic anhydride, and a trace of concentrated sulfuric acid for eight hours in a sealed tube at 100°. The crude product was recrystallized from ethanol. This monoacetyl derivative (V*b*) forms colorless thin leaflets melting at 198.7–199.3°.

Anal.—Calcd. for $C_{36}H_{22}O_7$: C, 76.31; H, 3.91; mol. wt. 566; CH_3CO-, 7.59. Calcd. for $C_{36}H_{24}O_7$: C, 76.05; H, 4.25; mol. wt. 568; CH_3CO-, 7.56; Found: C, 75.84; H, 4.31; mol. wt. 576 ± 25[7]; CH_3CO-, 7.49.

It was hydrolyzed by refluxing 0.2 Gm. in a mixture of 10 cc. 50% acetic acid and 1.5 cc. 10 *N* sulfuric acid, for six hours. The crude product was separated into two compounds by fractional crystallization from glacial acetic acid. The one less soluble melted at 250° and was found to be identical with compound V; the other (V*a*) melted at 232.8–234.9° and analyzed 77.70% C and 4.10% H. On acetylation, it again yielded the acetyl compound V*b*, melting at 199°. A mixture of compound V with V*a* melted at 235–245°. Compound V*a* is a dimorph of compound V.

The methyl ether (V*c*) was made by two different methods. First, it was prepared by the method of Freudenberg and Cohn (24), as follows: 2 cc. dimethyl sulfate and 2 cc. *N* sodium hydroxide were added to a solution of 0.50 Gm. of the acetyl derivative V*b* in 20 cc. acetone. After refluxing for two hours, an additional 0.5 cc. dimethyl sulfate and 0.5 cc. *N* sodium hydroxide were added, and the mixture was again refluxed for two hours. The acetone was then removed by distillation, 13 cc. 2.5 *N* sodium hydroxide added, and the precipitated oil washed with water. It became crystalline on rubbing with a little alcohol and melted at 199–203°. After crystallizations from ethanol, it melted at 209.7–210.9°. Besides the monomethyl ether (V*c*), which is the principal product, other compounds formed; however, the quantities were not sufficient for further examination. The monomethyl ether (V*c*) was also prepared by refluxing, for eight hours, a mixture of 0.09 Gm. of V, 0.07 Gm. of K_2CO_3 with 1.2 cc. acetone and 0.5 cc. methyl iodide. After two crystallizations from ethanol (0.1 Gm. in 1.4 cc.), the compound weighed 0.062 Gm. and melted at 210.8–211.3°.

Anal.—Calcd. for $C_{33}H_{22}O_6$: C, 78.06; H, 4.12; mol. wt. 538; CH_3O-, 5.66. Found: C, 77.92; H, 4.29; mol. wt. 537[6]; 500[7]; CH_3O-, 5.63.

On reduction of compound V, a dicarboxylic acid (V*d*) is formed. For this purpose, 0.55 Gm. of compound V, 0.22 Gm. zinc dust and 10 cc. acetic acid were refluxed for twelve hours. After crystallization from glacial acetic acid or from 30% ethanol, the reduction product (V*d*) melts at 278.1–279.6°. This dicarboxylic acid can also be recrystallized from ether: 0.1 Gm. is dissolved in 33 cc. pure ether and the filtrate evaporated to a volume of 7 cc. It is very soluble in acetone or ethanol at room temperature, and dissolves in 0.1 *N* sodium hydroxide, forming a colorless solution. Its solution in concentrated sulfuric acid is brownish yellow. Neither its acetyl derivative nor the methyl ether, prepared by the use of diazomethane, could be made to crystallize.

Anal.—Calcd. for $C_{34}H_{24}O_6$: C, 77.27; H, 4.54; mol. wt. 528; neutral. equiv. 264. Calcd. for $C_{34}H_{26}O_6$: C, 76.92; H, 4.91; mol. wt. 530; neutral. equiv. 265. Found: C, 76.74; H, 4.72; mol. wt. 486[6]; neutral. equiv. 265.

On decarboxylation of V*d* at 240° in quinoline, with basic copper carbonate as a catalyst, about 78 cc. CO_2 for 1 Gm. of V*d* was evolved. The oily compound could not be made to crystallize.

The quantity of the various compounds isolated from yellow phenolphthalein is shown in Table I.

TABLE I.—COMPOUNDS ISOLATED FROM YELLOW PHENOLPHTHALEIN[a]

	Yellow Phenolphthalein Monsanto Chemical Company ——Lot W-69-Y——		Yellow Phenolphthalein Prepared According to U. S. Patent 2,168,346, %	Phenolphthalein Residues from Chas. Pfizer & Co. ——Separation by——	
	Exp. A %	Exp. B %		Chromatography %	Molecular Distillation %
Phenolphthalein (I)	94.65	94.86	93.16	20.59	36.99
Fluoran (II)	0.25	0.27	0.32	2.97	2.28
Isophenolphthalein (III)	0.12	0.17	0.08	8.80	4.61
Phenolphthalin (IV)	0.87	0.94	...	...	...
Compound of m. p. 250° (V)	....	0.06	0.04	0.63	0.29
2-(4-Hydroxybenzoyl)-benzoic acid (VI)	0.005	...	0.10	...	...
Total of compounds isolated	95.89	96.30	93.70	32.99	44.17

[a] The figures represent the minimum quantity isolated.

Method for the Determination of Laxative Action.—The rhesus monkey (*Macacus rhesus*) has been found the most satisfactory for the biological assay of phenolphthalein because, of all laboratory animals, this species most closely approximates humans in its response to laxatives (5, 6, 25). The monkeys selected are healthy, mature animals, weighing 2.5 to 7 Kg. They are kept in individual cages, housed in a heated, well ventilated, glass-brick structure. Their diet consists of cereals, fresh vegetables and fruits, eggs, with the addition of 1 tablet of cod liver oil concentrate and 4 tablets of brewers' yeast. The animals become accustomed to their new surroundings in about six weeks. By this time, the stools should be consistently solid. Before the administration of the laxative substance, daily stool readings are taken, and only those animals are used that show solid stools over a period of at least three consecutive

days. At this time, the threshold dose for each animal is established.

As a standard for comparison with other laxative substances, phenolphthalein U. S. P. XII is used, because this product is chemically as pure as it is practicable to obtain. The substance to be tested is administered mixed with or hidden in some food the animals like, such as apple, banana, or orange. At times, the animals become suspicious of the food prepared in this way and refuse it or reject the part containing the drug. In such instances, it was found expedient to mix the dose to be administered with cooked oatmeal or rice, and offer it at the breakfast feeding. The animals are watched to see whether they consume the entire dose.

Phenolphthalein U. S. P. is administered in a dose sufficient to produce 1 to 4 soft stools over a period of not more than two days. The stool is designated as soft when its consistency permits it to pass through a one-half inch screen to the removable pan in the bottom of the cage. The minimum amount of phenolphthalein required to produce this result is considered the Unit Standard Dose (*U. S. D.*) for this individual animal. The dose is expressed in mg./Kg. proportion, the milligrams representing the dose of phenolphthalein, the kilograms the weight of the animal. This formula is followed by the number of soft stools passed over a two-day period. For instance, the Unit Standard Dose for animal No. 318 is 6.0 mg./Kg. (4S); that is, 6 mg. of phenolphthalein per kilogram weight will produce 4 soft stools in this particular animal. This standardization is checked at frequent intervals and, as a rule, shows little variation in mature animals.

The preparation to be tested for potency is administered in gradually increasing doses at about weekly intervals until the effects approximate closely those obtained with the Unit Standard Dose. The amount in mg./Kg. is the Minimum Effective Dose for that animal of the particular preparation. Thus

$$\text{Laxative Potency} = \frac{\text{Unit Standard Dose}}{\text{Minimum Effective Dose}}$$

The result is expressed in decimal fractions and is compared with phenolphthalein U. S. P., which has a value of 1.0 (P = 1.0). If, for instance, the Unit Standard Dose is 6.0, and the Minimum Effective Dose is 8.0, a laxative potency of 0.75 is obtained. This would indicate that the product tested has a laxative potency 0.25 less than the standard for phenolphthalein U. S. P. (P = 1.0).

The average response in 6 to 10 animals is taken as indicative of the potency of the product tested. These results are reproducible with a mean deviation of 10% for the average group. The laxative potencies of different compounds isolated from yellow phenolphthalein and their mixtures are tabulated in Table II.

TABLE II.—LAXATIVE POTENCIES

Name of Substance	Melting Point, ° C.	Number of Experiments	Number of Experiments with Laxative Response	Median Laxative Dose (*A. E. D.* 50)[a]	Average Potency
Phenolphthalein, U. S. P. XII	261–262	112	73	18.2	1.0
Phenolphthalein, yellow	256–260	552	436	7.3	2.5
Phenolphthalin	237.2–238.0	4	0	500	<0.01
Fluoran	182.5–183.2	3	0	600	<0.01
Isophenolphthalein	205.5–206.4	38	2	760	<0.01
2-(4-Hydroxybenzoyl)-benzoic acid	211–215 sl. dec.	9	2		<0.02
1-Hydroxyanthraquinone	193.4–194.2	6	2	100	<0.1
2-Hydroxyanthraquinone	310.5–311.4	5	0		<0.08
1,4-Dihydroxyanthraquinone or quinizarin	200.4–200.9	8	3		0.21
1,8-Dihydroxyanthraquinone or chrysazin	193.6–194.0	8	4	43.0	0.42
Mixture of 70% phenolphthalein, U. S. P. and 30% isophenolphthalein	213–242	4	4	7.5	0.67
Mixture of 98% phenolphthalein, U. S. P. and 2% 1-hydroxyanthraquinone	254–259	7	7	12.5	0.99
Mixture of 90% phenolpthalein, U. S. P. and 10% 2-hydroxyanthraquinone	238–255	9	9	11.4	0.97
Mixture of 95% phenolphthalein, U. S. P. and 5% 1,8-dihydroxyanthraquinone	252–258	6	6	8.1	1.03

[a] *A.E.D.* = average effective dose

Toxicity Test.—The Unit Standard Dose for eight healthy monkeys was determined. Yellow phenolphthalein mixed into cooked oatmeal was administered in doses one hundred times the *U. S. D.* for each animal. The animals were kept under constant observation for untoward manifestations, such as prostration, tremor, bloody stools, or other signs of toxic or irritant influence. The experiment was twice repeated at monthly intervals. The animals had diarrhea for two to three days, followed by scanty but formed stools. There were no signs of prostration or any other untoward effect. The animals took their food well and appeared to be

normal. At the end of the third experiment, 3 animals were sacrificed and examined. There was no evidence of congestion or other gross pathological changes in any of the internal organs.

SUMMARY

1. No monohydroxyanthraquinones could be detected in yellow phenolphthalein. The presence of polyhydroxyanthraquinones is very unlikely.

2. The compounds isolated up to now from yellow phenolphthalein are: phenolphthalein, fluoran, isophenolphthalein, phenolphthalin, a compound melting at 250°, and traces of 2-(4-hydroxybenzoyl)-benzoic acid. The compound melting at 250° has the probable formula of $C_{28}H_{19}O\left(\begin{matrix}C{=}O\\ \diagdown\\ -O\end{matrix}\right)_2(OH)$.

3. Yellow phenolphthalein was found about 2.5 times more active as a laxative in rhesus monkeys than phenolphthalein U. S. P.

4. Doses a hundred times greater than the average laxative dose of yellow phenolphthalein did not produce any toxic symptoms in rhesus monkeys.

5. The laxative action of compounds isolated from yellow phenolphthalein was determined, individually and in mixtures. The most active of these isolated compounds did not exceed the laxative potency of phenolphthalein U. S. P.

6. The reason for the greater laxative action of yellow phenolphthalein in comparison with phenolphthalein U. S. P. could not be established. Neither could the substances responsible for the yellow color of yellow phenolphthalein be isolated and identified.

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Book Review

Dynamic Aspects of Biochemistry. By ERNEST BALDWIN. The Macmillan Company, New York, 1947. xviii + 457 pp. 13.5 x 20.5 cm. Price $4.

Biochemistry has made significant advances in many of its phases during the last several years. Because it is such an active research field, textbook writers tend to freeze the information at a certain historical point and to emphasize chemical structure which is by nature more static.

Dr. Baldwin has performed a useful service to biochemistry in the publication of this book by separating purely organic structural matters from chemical reactions at the cellular level. Borrowing from biology, Dr. Baldwin very aptly classes the organic chemical material as morphologic in nature. This excellent book then limits itself to more dynamic problems of the behavior and function of the organic compounds in organized biological systems.

The natural starting point of discussing dynamic biochemistry is with the enzymes. The basic principles of enzyme properties and behavior are discussed in about 56 pages. Discussion of individual enzymes naturally follows.

Part II of the book is concerned with metabolic problems. The author proceeds from the general to the specific in a logical fashion. While conservative in outlook, the author frequently points out voids in our knowledge, a feature that makes the book stimulating reading.

Good indexing and the inclusion of all tables in the Table of Contents make this volume a useful tool to those interested in this supporting pharmaceutical science.

The Colorimetric Estimation of Digitalis*

By ORLO F. SWOAP

The Knudson and Dresbach procedure was used by the author a number of years ago, and this paper presents early experience gained with the method. The study reported covers a four-year period and compares results obtained on twenty digitalis tinctures and thirty-seven lots of a purified preparation of digitalis glycosides as tested by both the cat and colorimetric methods. As this work was done before the U. S. P. Reference Standard Digitalis was available, the values are expressed in terms of ouabain for the colorimetric method and in cat units for the biological cat method. The results emphasize the need for a different standard for each type of digitalis preparation tested.

THE STANDARDIZATION of digitalis and its preparations has been a question of much controversy for many years. A wide variety of both biological and chemical methods has been proposed from time to time, used for a while and then discarded as newer methods were proposed. No attempt will here be made to review completely the literature on this subject; but of the numerous chemical tests proposed for the digitalis glycosides it may be pointed out that the majority have involved a color reaction (1–8).

It is generally conceded that biological tests are better than chemical tests for determining the therapeutic value of digitalis preparations, as evidenced by the fact that five revisions of the U. S. P. have adopted biological methods for the standardization of official digitalis preparations. The change from frog to cat assay in the U. S. P. XII was made in the light of progress toward a more reliable method but is still criticized as not meeting ideal requirements. It is also generally recognized that this method is costly and time consuming, and extensive use is hampered by a shortage of cats. Consequently, research is still directed toward a more rapid chemical or colorimetric method of assay.

During the last two years, considerable attention has again been drawn to the Knudson and Dresbach (6) color method. This method is based on the original Baljet (4) reaction and is the only colorimetric method which has received much attention. A search of the literature indicates a wide divergence of opinion with regard to the reliability of the method. However, the method, as modified by Bell and Krantz (9) to use a photoelectric colorimeter for measuring colors, is being considered as a possible U. S. P. method for the assay of digitalis preparations.

The original Knudson and Dresbach (6) method was used in our laboratories a number of years ago as a check assay method before the U. S. P. XII cat assay method became official. At that time we were using the Magnus (10) modification of the Hatcher-Brody cat method as well as the official frog method, and found the color method of value in estimating potency and in checking various intermediate steps of manufacture. The purpose of this paper is to present our early experience gained with the method, which it is hoped may be of some value in the present study of the method. The work covers a four-year period and compares results obtained on twenty tinctures of digitalis and thirty-seven lots of a purified preparation of digitalis glycosides as tested by both the cat and colorimetric methods. As this work was done before the U. S. P. Reference Digitalis Standard was available, the values are expressed in terms of ouabain for the color method and in cat units for the biological cat method.

EXPERIMENTAL

The Knudson and Dresbach (6) colorimetric method used can be summarized as follows: A definite volume (2–5 cc., depending on activity) of tincture of digitalis is diluted with water to about 15 cc.; 2.5 cc. of a 10% solution of neutral lead acetate is added, mixed, and the solution diluted to 25 cc. After mixing thoroughly and allowing to stand a minute, the solution is filtered. This removes the color of the solution and, at most, the filtrate is only slightly yellow. The excess lead is removed by treating 12.5 cc. of this filtrate with 1.25 cc. of a 10% solution of Na_2HPO_4. This precipitates the lead, and the mixture is then diluted to 25 cc., mixed and filtered. The resulting filtrate is then ready for development of the color. Five cc. is then mixed with 5 cc. of the Knudson and Dresbach alkaline picrate solution (95 cc. of aqueous 1% purified picric acid solution + 5 cc. of 10% NaOH solution), set aside for twenty minutes to develop the color, and then compared with the standard in a Klett bio-colorimeter. Readings are taken in from twenty to thirty minutes after mixing, as the color tends to deepen on standing.

The purified digitalis glycoside preparation also used in our tests was prepared according to the

* Received Oct. 1, 1947, from the Research Laboratories, The Upjohn Company, Kalamazoo, Mich.
Presented to the Scientific Section, A. PH. A., Milwaukee meeting, August, 1947.

process of Hatcher and Haag (11). As these solutions are practically colorless, the above decolorization steps were omitted, and the color was developed directly on an aqueous dilution of the purified solution.

The standard used in our colorimeric work was a solution of ouabain, and the color was developed in the same manner as with the unknowns. Knudson and Dresbach (6) used a standard solution of crystalline ouabain containing 0.266 mg. in 5 cc. of water. This solution, when mixed with 5 cc. of the alkaline picrate solution, was stated by them to give a color equal in intensity to that of 0.5 cc. of a standard tincture assayed by the Hatcher cat method when treated in a similar manner and diluted to 10 cc. volume with water. In other words, 0.266 mg. ouabain is considered equal to half a cat unit as expressed by Hatcher's method, or 1 cat unit (color) = 0.532 mg. ouabain. Hatcher's cat unit (biological) = 0.1 mg. ouabain, so that Knudson and Dresbach's color standard was therefore an entirely empirical one based upon their "standard" tincture.

Preliminary experiments with several tinctures of digitalis following the Knudson and Dresbach method in detail gave results in "color" cat units per cc. of tincture, which were uniformly much higher than those obtained by the cat assay itself. Thus, it appeared that the Knudson and Dresbach standard amount of ouabain in the "color" cat unit (1 "color" cat unit = 0.532 mg. ouabain) was too small, at least for use in this laboratory. It was therefore decided to continue testing digitalis tinctures and to determine for our laboratory the amount of ouabain in a "color" cat unit.

In order to compare the colors at a level corresponding to that used by Knudson and Dresbach, our standard ouabain solution was first made up to contain 0.0532 mg./cc. For simplicity of calculation, however, we then changed this to 0.05 mg./cc. A varying amount of unknown was then used in order to make colorimetric readings correspond as closely as possible to those of the standard. The amount of ouabain contained in each cc. of unknown was then calculated by using the formula:

$$\text{Mg. ouabain/cc. of unknown} = S/U \times \text{mg. ouabain/cc. of standard} \times \text{dilution of unknown}$$

where

S = colorimetric reading of standard in mm.
U = colorimetric reading of unknown in mm.

The amount of ouabain in a "color" cat unit was then found by dividing this value of mg. ouabain/cc. of unknown by the biological cat units/cc. of unknown, as determined in this laboratory by the above-mentioned Magnus modification of the Hatcher-Brody cat method (10).

Twenty tinctures of digitalis were tested over a period of about four years, and results are summarized in Table I, which shows the "color" cat unit for digitalis tincture to average 0.970 mg. ouabain. For the method to be reliable, individual values should show uniformity within the experimental error of the method, but as a biological unit was used to derive them it is to be expected that they should show no greater uniformity than would be expected from the biological method itself. An examination of the results shows that 96% of the individual values are within ±15% of this mean (75% are within ± 10% of the mean) and only 4%, or one individual value, is as much as 19% from the mean. In this instance, the tincture (No. OFS-3-50) was about six years old and had originally tested 1.15, 1.03, and 1.12 biological cat units per cc. The six-year reassay showed values of 1.15, 0.928, and 0.716 biological cat units per cc. The values were averaged for calculation of the mg. ouabain per "color" cat unit value.

TABLE I.—TINCTURE DIGITALIS

Preparation Number	Biological		Colorimetric					
	C. U./ Cc. Tr.	No. Cats Used	St'd.	Un.	Mg. Oua./Cc. Std.	Dilu. of Un.	Mg. Oua. Eqvt./ Cc. Tr.	Ratio Mg. Oua./ C. U. (Color)
67420	1.386	4	15	12.4	0.0532	1–20	1.287	0.929
67709	1.712	6	15	18.8	0.05	1–40	1.596	0.932
770767	1.650	4	20	13.8	0.05	1–20	1.449	0.878
B-3880	1.178	2	20	16.5	0.05	1–20	1.212	1.029
771632	1.503	4	20	13.4	0.05	1–20	1.492	0.993
772207	1.488	4	20	13.6	0.05	1–20	1.470	0.988
773267	1.500	4	20	22.3	0.05	1–33.3	1.495	0.997
773267	1.500	4	20	17.6	0.05	1–25	1.420	0.947
773267	1.500	4	20	22.4	0.05	1–33.3	1.488	0.992
773895	1.502	4	20	18.2	0.05	1–25	1.374	0.915
774581	1.559	4	20	19.1	0.05	1–25	1.309	0.840
774830	1.468	4	20	18.5	0.05	1–25	1.351	0.920
775574	1.418	4	20	19.7	0.05	1–25	1.269	0.895
L.A.	0.948	3	20	19.3	0.05	1–20	1.036	1.093
775899	1.529	4	20	18.9	0.05	1–25	1.323	0.865
776867	1.242	3	20	18.4	0.05	1–20	1.087	0.875
776934	1.277	4	20	18.8	0.05	1–25	1.330	1.042
OFS-3-50	1.015	6	20	17.1	0.05	1–20	1.170	1.153
OFS-3-51	1.572	3	20	18.9	0.05	1–25	1.323	0.842
777468	1.435	8	20	17.7	0.05	1–25	1.413	0.985
778309	1.479	4	20	16.8	0.05	1–25	1.488	1.006
778867	1.742	6	20	18.9	0.05	1–33.3	1.763	1.012
778867	1.742	6	20	21.2	0.05	1–40	1.886	1.083
778867	1.742	6	20	18.1	0.05	1–33.3	1.842	1.057
							Av.	0.970

During the same period of time, thirty-seven lots of a purified preparation of digitalis glycosides prepared by the process of Hatcher and Haag (11) were tested in the same manner. The results, summarized in Table II, show an average of 0.532 mg. ouabain per cat unit, which, by coincidence, is identical with Knudson and Dresbach's value for tincture of digitalis. An examination of Table II shows that 82% of the individual values are within ±20% of the mean (58% are within ±10% of the mean), and only 5% or two individual values differ by more than ±30% of the mean. In both of these instances the cat assay was based on only two cats. These results vary more than those obtained with the tincture, but it will be noted that fewer cats were used for almost every assay.

The ten values from Table II derived from biological assays where 6 to 10 cats were used on each assay show an average of 0.515 mg. ouabain per cat unit. Seventy per cent of these ten values are within ±10% of the mean, 90% are within ±12% of the mean, and only one value is as much as 32% from the mean. This value is based on a biological assay of 7 cats with a standard error of 10.5%. Excluding this value the average of the remaining nine values is 0.497 mg. ouabain per cat unit, with all values within ±8.5% of the mean.

From the above we may conclude that if we adopt the nearest whole number, i.e., 1.0 mg. ouabain per "color" cat unit for tinctures and 0.5 mg. ouabain per "color" cat unit for purified preparations of digitalis, we may expect actual cat assays (biological) to agree with these values within the accepted experimental error.

We have at various times checked the Hatcher standard of 0.1 mg. ouabain per cat unit within experimental error, so in using ouabain as a standard for the color assay it is evident that 0.1 mg. ouabain also equals one "color" cat unit for ouabain. One lot of Strophanthin powder (Merck) was compared by the cat and colorimetric methods. The cat method gave 5.881 cat units per mg. Strophanthin

TABLE II.—PURIFIED PREPARATIONS OF DIGITALIS GLYCOSIDES

Preparation Number	Biological		Colorimetric					Ratio Mg. Oua./C. U. (Color)[a]
	C. U./ Cc. Tr.	No. Cats Used	Std.	Un.	Mg. Oua./Cc. Std.	Dilu. of Un.	Mg. Oua. Eqvt./ Cc. Soln.	
6851-2	0.671	3	20	12.8	0.0532	1–5	0.415	0.618
6851-3	0.828	3	20	16.2	0.0532	1–5	0.329	0.397
6851-4	0.747	3	20	16.0	0.0532	1–5	0.333	0.446
6851-6B	0.624	3	20	17.9	0.05	1–5	0.279	0.447
6851-10 (1)	3.057	2	20	17.0	0.05	1–30	1.764	0.577
6851-9	0.587	6	20	15.9	0.05	1–5	0.315	0.537
6851-10 (2)	0.650	6	20	14.6	0.05	1–5	0.343	0.528
6851-6A	1.235	7	20	17.8	0.05	1–10	0.562	0.455
6851-11A1	3.190	2	20	24.1	0.05	1–30	1.245	0.390
6851-11B1	1.555	2	20	19.8	0.05	1–15	0.758	0.487
6851-12A1	2.957	2	20	20.4	0.05	1–30	1.470	0.497
6851-12B1	1.727	2	20	20.2	0.05	1–20	0.990	0.573
6851-11A2	0.560	2	20	22.4	0.05	1–5	0.223	0.398
6851-11B2	0.392	2	20	19.4	0.05	1–5	0.258	0.658
6851-12A2	0.511	2	20	19.5	0.05	1–5	0.257	0.503
6851-12B2	0.463	2	20	17.5	0.05	1–5	0.286	0.618
6851-13 (1)	3.253	2	20	18.4	0.05	1–30	1.631	0.501
6851-14 (1)	1.786	2	20	14.8	0.05	1–20	1.351	0.756
6851-13 (2)	1.006	2	20	18.0	0.05	1–10	0.556	0.553
6851-14 (2)	1.489	2	20	16.5	0.05	1–15	0.909	0.610
6851-14 (3)	1.273	2	20	15.6	0.05	1–10	0.641	0.504
6851-15 (1)	0.675	3	20	14.0	0.05	1–5	0.357	0.529
6851-15 (2)	0.522	7	20	17.9	0.05	1–5	0.279	0.534
6851-16	0.544	7	20	20.3	0.05	1–7.5	0.369	0.678
12907 (1)	0.894	3	20	15.5	0.05	1–7.5	0.484	0.541
12907 (2)	0.691	7	20	14.3	0.05	1–5	0.350	0.507
12977-8 (1)	0.635	3	20	17.1	0.05	1–6	0.351	0.553
6851-12A13A	0.540	2	20	19.0	0.05	1–5	0.263	0.487
12977-8 (2)	0.659	7	20	15.8	0.05	1–5	0.317	0.481
12977-8 (3)	0.560	8	20	18.1	0.05	1–5	0.276	0.493
13153 (1)	1.013	3	20	20.8	0.05	1–10	0.481	0.475
13153-(2)	0.583	6	20	17.9	0.05	1–5	0.279	0.479
13030	0.593	3	20	15.5	0.05	1–5	0.323	0.545
13030	0.593	3	20	20.2	0.05	1–7.5	0.371	0.626
13270	0.597	10	20	18.4	0.05	1–5	0.272	0.456
13231	0.535	2	20	18.3	0.05	1–5	0.273	0.510
12861	0.494	2	20	18.1	0.05	1–5	0.276	0.559
13142	0.392	2	20	18.4	0.05	1–5	0.272	0.694

Total average, 0.532.
[a] Average 10 runs using 6–10 cats, 0.515.

TABLE III.—COMPARISON OF STANDARDS

Substance	Biol. Cat Unit	Color C. U. Equivalent
Tincture of Digitalis	1.0 cc. tincture	1.0 mg. ouabain
Purified Solutions of Digitalis Glycosides	1.0 cc. solution	0.5 mg. ouabain
Strophanthin	0.216 mg. strophanthin	0.143 mg. ouabain
Ouabain	0.100 mg. ouabain	0.100 mg. ouabain

and the colorimetric method gave 0.84 mg. ouabain per mg. Strophanthin. Thus, for Strophanthin, we find that 0.84/5.881 = 0.143 mg. ouabain per cat unit (color).

The above results emphasize the need for a different standard amount of ouabain for each type of digitalis preparation tested by the colorimetric method if a conversion is to be made for the estimation of biological cat units. A comparison of biological cat units and colorimetric cat unit equivalents, as determined for the preparations tested, is given in Table III.

It will be noted from Table III that tincture of digitalis gives a much higher color equivalent of ouabain than the other preparations and therefore must contain some inactive substance which produces a color by this method. It is well recognized that the Baljet reaction is not specific for digitalis glycosides. For instance, dextrose also gives this reaction. However, Lendle and Schmelzer (12), Neumann (13), and Bell and Krantz (9) have shown that this would not interfere with normal colorimetric assays of tincture of digitalis. The biologically inactive glycoside "diginin" isolated by Karrer (14) is reported by Hagemeier (15) to have the greatest color activity with alkaline picrate solution of any preparation tested. However, Bell, Carr, and Krantz (16) in an investigation of diginin report that it produces a very weak color. From the work of these investigators we may conclude that the presence in digitalis of varying amounts of diginin, or other as yet unidentified substances, may be contributory to errors in the colorimetric assay of tincture of digitalis.

SUMMARY

Some early experience with the Knudson and Dresbach colorimetric method for the assay of digitalis preparations is presented.

The work covers a four-year period and compares results obtained on twenty tinctures of digitalis and on thirty-seven lots of a purified preparation of digitalis glycosides as tested by both the cat and colorimetric methods. As this work was done before the U. S. P. Reference Standard Digitalis was available, the values are expressed in terms of ouabain for the color method and in cat units for the biological cat method.

The results emphasize the need for a different standard amount of ouabain for each type of digitalis preparation tested by the colorimetric method if a conversion is to be made for the estimation of biological cat units.

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Book Review

Pharmacology and Experimental Therapeutics. A Survey for 1941–1946. By HAMILTON H. ANDERSON, FUMIKO MURAYAMA, and BENEDICT E. ABREU. University of California Press, Berkeley and Los Angeles, 1947. viii + 368 pp. 15 x 23.5 cm. Price $6.50.

Quantitative pharmacological data are often very difficult to find in the literature. During the year 1941–1946, many data of this character were accumulated, but often they are not too easily accessible. The authors of this book have brought together this information. The effort has been to relate dosage to specific effect. *In vitro* and *in vivo* effects and activities are recorded for several species and especially in man. Blood and other tissue levels of a drug are listed as well as biological and chemical tests used to identify drugs and poisons in body fluids and tissues. Common synonyms for the drugs and poisons described are usually listed. The substances are listed alphabetically under their most widely accepted names. Indexing of synonyms would have been very helpful in locating some of the substances.

The compilers are to be congratulated on producing a very useful tool, especially for the research worker.

The Antisepticity of Various Ointments Containing a Germicide of the Quaternary Ammonium Type*

By FANCHON HART† and C. LEE HUYCK‡, §

A review of the literature pertaining to the effect of various ointment bases on the antiseptic activity of water-soluble germicides is presented. The effect of various ointment bases on the antiseptic activity of the water-soluble detergent germicide of the quarternary ammonium type has been studied. This paper includes formulas and directions for the preparation of the ointments as well as the results of the antisepticity tests as carried out according to the Food and Drug Administration wet and dry filter-paper and cup-plate methods.

MANY DATA have been reported concerning the effect of various ointment bases on the antiseptic activity of water-soluble germicides but few have been reported on the effect of ointment bases on the antiseptic activity of the water-soluble detergent germicides of the quaternary ammonium type.

Husa and Radin (1) tested the effect of variation in the base on the antiseptic value of phenol ointment by the serum agar cup-plate test of Reddish (2). Tests made on 5 per cent phenol ointment containing water, anhydrous lanolin, and petrolatum showed that the presence of a small proportion of water was unimportant but that the relative proportions of anhydrous lanolin and petrolatum were the deciding factors in determining antiseptic properties. Ointments containing 5 per cent phenol in a base of petrolatum and anhydrous lanolin showed antiseptic properties when the proportion of anhydrous lanolin in the base was 23 to 25.5 per cent. The incorporation of 5 per cent yellow wax reduced the antiseptic value. Tests with individual bases showed that hydrous lanolin, aquaphor, and benzoinated lard gave antiseptic ointments with lower percentages of phenol. The authors recommended an ointment containing 2 per cent phenol, 24.5 per cent wool fat and 73.5 per cent petrolatum for inclusion in the United States Pharmacopœia since this ointment showed definite antiseptic properties.

Foley and Lee (3) studied the antiseptic action of various commonly known chemicals in fatty, nonfatty, absorption, and water-soluble jelly bases by the agar-plate method using *Staphylococcus aureus* as the test organism. Boric acid was negatively bacteriostatic in the hydrophilic bases in which a large amount of water was present. Ammoniated mercury was consistently active in all the bases tested, but most active in the vanishing cream base. Yellow mercuric oxide gave positive activity in all the bases tested with the largest zone of inhibition in vanishing cream. Calomel was, in general, active in hydrophilic bases but equally active in anhydrous wool fat. Phenol was inactive in the usual fatty bases but showed its best activity in the Seltzer base No. 4 (sodium lauryl sulfate, cetyl, stearyl and oleyl alcohols, ceresin and heavy liquid petrolatum). Iodine behaved very well in all the fatty bases but was inactive in vehicles containing large amounts of water. Benzoic and salicylic acids (Whitfield's ointment) were more active in water-containing bases. The authors concluded that the nature of the chemical or medicament incorporated in the base has as much to do with the bacteriostatic action of the ointment as the vehicle.

Busse (4) reviewed ointment bases as carriers for antiseptics and after analyzing the results drew the following conclusions: (*a*) There is as yet no ointment base which can serve as a universal ointment base. (*b*) The conclusion, by G. F. Reddish, "that the antiseptic value of an ointment could not be told by the antiseptic value of its constituents" still holds true. (*c*) The advantages claimed for water-containing ointment bases as vehicles for antiseptics over the greasy type bases are not justified unless on an individual basis. (*d*) The chemical and physical nature of the antiseptic which is incorporated in the base has as much or perhaps more to do in determining the final bactericidal action of the product as the composition of the vehicle in which it has been incorporated.

Di-isobutyl cresoxyethoxy ethyl dimethyl benzyl ammonium chloride (5) commonly known as Hyamine 10X is a quaternary ammonium salt with high bactericidal efficiency and low toxicity. It is readily soluble in water, forming stable, odorless solutions with surface-active properties It has the following structure:

$$\left[C_8H_{17}C_6H_3CH_3OC_2H_4OC_2H_4N(CH_3)_2\right]^{+}_{\left|\right.}\;\overset{-}{\underset{CH_2C_6H_5}{}}Cl$$

* Received September 29, 1947, from the Columbia University College of Pharmacy, New York, N. Y.
Presented to the Scientific Section, A. PH. A., Milwaukee meeting, August, 1947.
† Columbia University College of Pharmacy.
‡ Howard College, Birmingham, Alabama.
§ The authors are indebted to Rohm and Haas Co., Philadelphia, Pa., for supplying the Hyamine 10X for this investigation.

Solutions of Hyamine 10X are compatible with rubber, plastics, glass, and ceramic ware. Since it is a wetting agent, solutions thoroughly wet metal surfaces and give water full opportunity to produce corrosion. Steel containers therefore should be lacquered. Hyamine 10X is compatible with alkaline detergents such as caustic soda, soda ash, and trisodium phosphate but it is incompatible with phosphates other than trisodium phosphate. Because it is cation active, it is incompatible with anion active soaps and wetting agents forming inactive stearates and palmitates.

Bactericidally effective dilutions, when tested by the Food and Drug Administration method (6) at 20° using *S. aureus* as test organism, are 1:6000 in five minutes and 1:6500 in ten minutes. A 25 per cent solution of Hyamine 10X has a phenol coefficient of 40 to 50 using *Eberthella typhi* and 90 to 100 using *S. aureus*.

As for toxicity the LD_{50} or the average dose in terms of pure crystals necessary to kill one-half of the group of animals is 15 mg./m. for mice and 800 mg./Kg. for rats. When given subcutaneously it is 3.8 mg./m. for mice and 100 mg./Kg. for rats.

EXPERIMENTAL

Hyamine 10X was incorporated in (1) Modified Hydrophilic Base, (2) White Ointment Base U. S. P. XIII, (3) Modified Hydrophilic Petrolatum, (4) Phenol Ointment Base U. S. P. XIII, (5) Burn Ointment Base of the Pharmaceutical Recipe Book in 2, 1, and 0.1% concentrations.

The complete formulas and directions for preparation of the ointments containing 2% Hyamine 10X are given below. In varying the percentage of germicide, slight adjustment to 100% was made with distilled water in No. 1, white petrolatum in Nos. 2, 3, and 4 and white petrolatum, wool fat, and distilled water in equal amounts in No. 5.

Formula 1

Hyamine 10X	2%
Glycerin U. S. P.	12%
Stearyl Alcohol U. S. P.	25%
White Petrolatum U. S. P.	25%
Distilled Water U. S. P.	36%

Directions: Melt the stearyl alcohol and the white petrolatum on a water bath, adjust the melted mixture to about 75° and add the other ingredients previously dissolved in the distilled water warmed to 75°. Stir the mixture until it congeals.

Formula 2

Hyamine 10X	2%
White Wax U. S. P.	5%
Wool Fat U. S. P.	5%
White Petrolatum U. S. P.	88%

Directions: Melt the white wax in a suitable container on a water bath, add the other ingredients, warm until they are liquefied, then discontinue the heat and stir the mixture until it congeals.

Formula 3

Hyamine 10X	2%
Stearyl Alcohol U. S. P.	3%
White Wax U. S. P.	8%
Wool Fat U. S. P.	16%
White Petrolatum U. S. P.	71%

Directions: Melt the stearyl alcohol, white wax, wool fat, and white petrolatum together on a water bath. Add the Hyamine 10X and stir the mixture until it congeals.

Formula 4

Hyamine 10X	2%
Glycerin U. S. P.	2%
Wool Fat U. S. P.	4.85%
White Wax U. S. P.	4.85%
White Petrolatum U. S. P.	86.3%

Directions: Melt the white wax in a suitable container on a water bath, add the other ingredients. Warm until they are liquefied, discontinue the heat and stir until the mixture congeals.

Formula 5

Hyamine 10X	2%
White Petrolatum U. S. P.	32.67%
Wool Fat U. S. P.	32.67%
Distilled Water U. S. P.	32.67%

Directions: Melt the white petrolatum and wool fat. Dissolve the Hyamine 10X in distilled water and bring it to the temperature of the fats. Incorporate the water in the fats and stir until the mixture congeals.

Difficulty was encountered in obtaining complete absorption of the germicidal solution by the wool fat and petrolatum in Formula 5. This ointment was found to be unstable at room temperature.

Tests for Antisepticity of the Ointments.—Twenty-four-hour cultures of *S. aureus* No. 209 were used as the test organism and the tests were conducted according to the standard method (6) at 20°. A 1 to 90 solution of phenol killed the organism in ten minutes but not in five minutes at 20°. A 1 to 100 solution of phenol did not kill the organism in five, ten, or fifteen minutes.

Five-tenths-centimeter pieces of Whatman filter paper No. 2 were sterilized and transferred to the twenty-four-hour broth culture of *S. aureus* No. 209 after which they were placed in each of the ointments for five, ten, and fifteen minutes and subsequently transferred to tubes of 10 cc. of sterile broth. All the ointment adhering to the filter papers was first removed with a sterile needle and the filter paper was then transferred to a second sterile-broth tube. Both tubes were then incubated for twenty-four and forty-eight hours at 37°. At the end of forty-eight hours one loopful of the incubated liquid was transferred to 10 cc. of fluid thioglycollate medium and incubated for twenty-four hours at 37°.

The same procedure was followed for the dry filter-paper method in which case the filter paper squares were first impregnated with the test culture and dried for forty-eight hours in a sterile Petri dish at 37° before they were placed in the ointments.

TABLE I.—RESULTS OF INCUBATION OF FLUID THIOGLYCOLLATE TRANSFER IN THE ANTISEPTICITY TESTS OF HYAMINE 10X OINTMENTS BY THE WET AND DRY FILTER-PAPER METHODS

No.	Base	Hyamine 10X, %	Growth of *S. aureus* After 5 Min.	Contact with Ointment for: 10 Min.	15 Min.
1	Modified Hydrophilic	2	Negative	Negative	Negative
1	Modified Hydrophilic	1	Negative	Negative	Negative
1	Modified Hydrophilic	0.1	Positive	Positive	Positive
2	White Ointment U. S. P.	2	Negative	Negative	Negative
2	White Ointment U. S. P.	1	Positive	Positive	Positive
2	White Ointment U. S. P.	0.1	Positive	Positive	Positive
3	Modified Hydrophilic Petrolatum	2	Negative	Negative	Negative
3	Modified Hydrophilic Petrolatum	1	Negative	Negative	Negative
3	Modified Hydrophilic Petrolatum	0.1	Negative	Negative	Negative
4	Phenol Ointment Base	2	Negative	Negative	Negative
4	Phenol Ointment Base	1	Positive	Positive	Positive
4	Phenol Ointment Base	0.1	Positive	Positive	Positive
5	Burn Ointment Base P. R. B.	2	Negative	Negative	Negative
5	Burn Ointment Base P. R. B.	1	Negative	Negative	Negative
5	Burn Ointment Base P. R. B.	0.1	Positive	Positive	Positive

TABLE II.—RESULTS OF THE MODIFIED AGAR CUP-PLATE TEST FOR ANTISEPTICITY OF OINTMENTS CONTAINING HYAMINE 10X WHEN TESTED AGAINST *S. aureus*.

Number	Base	Hyamine 10X %	Bactericidal Zone Mm.	Diffusion Mm.
1	Modified Hydrophilic	2	3	8
1	Modified Hydrophilic	1	3	18
1	Modified Hydrophilic	0.1	1.5	1.5
2	White Ointment U. S. P.	2	3	3
2	White Ointment U. S. P.	1	2	2
2	White Ointment U. S. P.	0.1	0	2
3	Modified Hydrophilic Petrolatum	2	2	6
3	Modified Hydrophilic Petrolatum	1	3	6
3	Modified Hydrophilic Petrolatum	0.1	3	3
4	Phenol Ointment Base	2	3	5
4	Phenol Ointment Base	1	2	2
4	Phenol Ointment Base	0.1	0	0
5	Burn Ointment Base P. R. B.	2	7	7
5	Burn Ointment Base P. R. B.	1	0	12
5	Burn Ointment Base P. R. B.	0.1	4	4

Since the results of the wet and dry filter-paper methods are the same, they are summarized in Table I.

Table I shows that 1% and 2% Hyamine 10X incorporated in modified hydrophilic base are antiseptic. One-tenth per cent Hyamine 10X in modified hydrophilic base is not antiseptic. White ointment base U. S. P. requires 2% Hyamine 10X to be antiseptic. Hyamine 10X is antiseptic in modified Hydrophilic Petrolatum in 0.1%, 1%, and 2% concentrations. Phenol ointment base requires 2% Hyamine 10X to be antiseptic while Burn Ointment base of the Pharmaceutical Recipe Book requires 1% Hyamine 10X to produce any antiseptic action.

The ointments were tested by the agar cup-plate method (6) at 20 degrees with *S. aureus* as test organism. Pieces of all zones showing activity of the ointments were transferred to fluid thioglycollate medium for confirmation of bactericidal or bacteriostatic activity. In regard to this test we quote Bartlett (7) "We do not feel that the Agar Cup-Plate Test is satisfactory as a measure of germicidal activity. The agar cup-plate test measures primarily the ability of the active ingredient in the ointment to diffuse through agar, so that in this test you are measuring diffusion rate rather than germicidal activity. Quaternaries in general do not diffuse very readily through agar and in most cases will give only narrow zone of inhibition. Nevertheless other tests which measure only germicidal activity show an exceptionally high degree of efficiency." For this reason the diffusion of the ointments was checked. The results of the agar cup-plate test are recorded in Table II.

Table II shows that diffusion is characteristic of the base. Modified hydrophilic ointment base containing stearyl alcohol, glycerin, white petrolatum and distilled water, and Burn ointment base of the Pharmaceutical Recipe Book containing equal parts of white petrolatum, wool fat, and distilled water appear to favor diffusion. Generally an increase in the amount of germicide increases the bactericidal action of the ointment.

Results of the Antisepticity Test Compared.—Modified Hydrophilic Base containing 1% Hyamine 10X is bactericidal by both the wet and dry filter-paper methods and by the agar cup-plate method The hydrophilic base containing 0.1% Hyamine 10X is not bactericidal by the wet and dry filter-paper methods but is bactericidal by the agar cup-plate method. White ointment base with 2% Hy-

amine 10X is bactericidal by both the wet and dry filter-paper methods and by the agar cup-plate test. White ointment base containing 1% Hyamine 10X is not bactericidal by the wet and dry filter-paper methods but is bactericidal by the agar cup-plate test. Modified Hydrophilic Petrolatum containing 0.1% Hyamine 10X is bactericidal by both the wet and dry filter-paper methods and by the agar cup-plate method. Phenol Ointment Base containing 1% Hyamine 10X is not bactericidal by the wet and dry filter-paper methods but is bactericidal by the agar cup-plate method. Burn Ointment Base with 0.1% Hyamine 10X is not bactericidal by the wet and dry filter-paper methods but is bactericidal by the agar cup-plate test.

The results of the agar cup-plate test agree with the results of the wet and dry filter-paper methods in the case of White Ointment Base containing 0.1% Hyamine 10X and Phenol Ointment Base containing 0.1% Hyamine 10X but in the case of Burn Ointment Base containing 1% Hyamine 10X there is a discrepancy in that the ointment failed to pass the bactericidal confirmation test.

DISCUSSION

Our results are in general agreement with the results of Husa and Radin (1) that water is not a necessary constituent for the production of antisepticity in ointments. The conclusion of Foley and Lee (3) and Busse (4), that the nature of the chemical incorporated in the base has as much to do with the bacteriostatic action of the ointment as the vehicle, appears to be well founded.

The wet and the dry filter-paper methods of testing the antisepticity of ointments gave consistently lower results than the agar cup-plate method. Klarman and Wright (8) have pointed out that the filter-paper method gives a lower result for the performance of a quaternary ammonium compound than other methods of testing germicides. In general, an increase in the amount of germicide increases the bactericidal action of the ointment.

As previously pointed out, Burn Ointment Base of the Pharmaceutical Recipe Book is not recommended as a vehicle for Hyamine 10X because of the difficulty encountered in its manufacture and because of its instability when stored at room temperature.

SUMMARY AND CONCLUSIONS

The quaternary ammonium compound commonly known as Hyamine 10X was incorporated in five different ointment bases in 2%, 1%, and 0.1% concentrations and tested for antisepticity by the wet and dry filter-paper methods and by the agar cup-plate test.

On the basis of the results obtained by the wet and dry filter-paper methods, modified Hydrophilic Petrolatum Base was found to be the best base. Burn Ointment Base of the Pharmaceutical Recipe Book containing equal parts of white petrolatum, wool fat, and distilled water as well as the modified hydrophilic base containing 12% glycerin, 25% stearyl alcohol, 25% white petrolatum, and 36% distilled water were of equal value as the next best base for Hyamine 10X. Burn Ointment Base, however, is not recommended as a vehicle for Hyamine 10X because of its instability.

Generally an increase in the amount of quaternary germicide present in the base increases the bactericidal action of the ointment.

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Book Review

Essentials of Pharmacology. By FRANCES K. OLDHAM, F. E. KELSEY, and E. M. K. GEILING. J. B. Lippincott Company, Philadelphia, 1947. xiv + 440 pp. 12 x 19.5 cm. Price $5.

Essentials of Pharmacology is a short text on elementary pharmacology designed by the authors to meet the needs of medical students. The authors have succeeded in condensing pharmacology into a remarkably few pages, but at times the reviewer believes it to be too brief. The addition of brief, practical directions for the clinical use for drugs in a fashion similar to that of *New and Nonofficial Remedies* would have helped considerably.

The drugs covered in this book are classified according to a very logical plan and the chief characteristics of the category are given. Under each drug appears a brief description of its action, administration, toxicity, and clinical uses. One of the important items frequently missed is the essential elimination and detoxification, i.e., epinephrine.

At the end of each section is a list of principal drugs with their doses. It is unfortunate that the authors have seen fit to include no National Formulary drugs although some N. F. drugs are included and accredited to N. N. R. The National Formulary plays too important a role in modern therapy and drug standardization to be completely overlooked.

As useful as this brief text is, it is difficult to believe that, without supplementation, future physicians will be adequately trained by using this text alone; certainly it is not completely adequate for pharmacists.

A Comparative Study of *Salix* Species Grown in Egypt*,†

By IBRAHIM R. FAHMY‡ and ISMAIL A. ABDEL-LATIF§

The taxonomical characters of the three Egyptian willows—*Salix babylonica* (Linné), *Salix Safsaf* (Forsk.), and *Salix tetrasperma* (Roxb.)—have been studied. The pharmacognostical study of the barks and leaves of the three species has been carried out. The active bitter constituent of the barks and leaves has been isolated and was proved to be the glycoside salicin.

THIS research was planned as a contribution toward a study of the morphology and histology of the leaves and barks of *Salix* species grown in Egypt, with a view to determining the characteristics by which these species can be distinguished; and a chemical investigation of both leaves and barks with the object of isolating, identifying, and determining the yield of their active bitter principles.

BOTANICAL STUDY

Salix Species Grown in Egypt.—There are three *Salix* species widely distributed throughout the Nile valley (1). They have been grown since the days of the Ancient Egyptians, to whom they were sacred (2). The Latin names of these species as well as their English and Arabic synonyms (3–7) are as follows:

1. *Salix babylonica* (Linné):
   Weeping Willow (English)
   Safsaf Om Ashōōr (Arabic)
2. *Salix Safsaf* (Forsk.):
   Salix subserrata (Willd.)
   Jordan Willow (English)
   Safsaf Balady (Arabic)
3. *Salix tetrasperma* (Roxb.):
   Safsaf Rumy (Arabic)

Description of Plants.—The three *Salix* trees behave similarly in being confined to the neighborhood of water and growing rapidly in damp places and on the banks of rivers and canals.

Salix babylonica (Linné).—It is indigenous to Caucasus and North Asia. It is widely distributed in the Mediterranian region, and is cultivated everywhere in Egypt. The tree is slender, about 6 to 10 meters high, but the long recurved drooping branches generally reach the surface of the stream, on the bank of which the tree is growing (Fig. 1).

Salix Safsaf (Forsk.).—It is indigenous to North and tropical Africa. Widely distributed throughout Egypt and the Sudan. The tree is moderate in size, about 5 to 8 meters high and profusely branched. The branches are stout, straight, and not drooping (Fig. 2), thus distinguishing it from *Salix babylonica*.

Fig. 1.—*Salix babylonica* Tree. Medicinal Plant Garden, College of Pharmacy, Fouad I University, Cairo, Egypt.

Salix tetrasperma (Roxb.).—It is indigenous to east India, Malaya, Singapore, Java, and Sumatra. This tree is larger and stouter than the other two species. It is about 10 to 12 meters in height and has straight branches (Fig. 3).

Leaves.—The leaves of the three species are simple, lanceolate, short petiolated, stipulate, deciduous, and fall in winter except those of *Salix tetrasperma* which is an evergreen plant. The three species can be readily differentiated by the different sizes of their leaves (Fig. 4).

Flowers.—The plants are dioecious, flowering from December to April. The flowers occur in catkins and appear before the leaves in *S. babylonica* and *S. Safsaf*. All plants of these two species were found to be female, while those of *S. tetrasperma* are all male. However, herbaria of male plants of

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* Abstract of thesis offered in partial fulfillment of the requirements for the Degree of Master of Pharmacy, Fouad I University, February 1943, Cairo, Egypt.

† Presented to the Scientific Section, A. PH. A., Milwaukee meeting, August, 1947.

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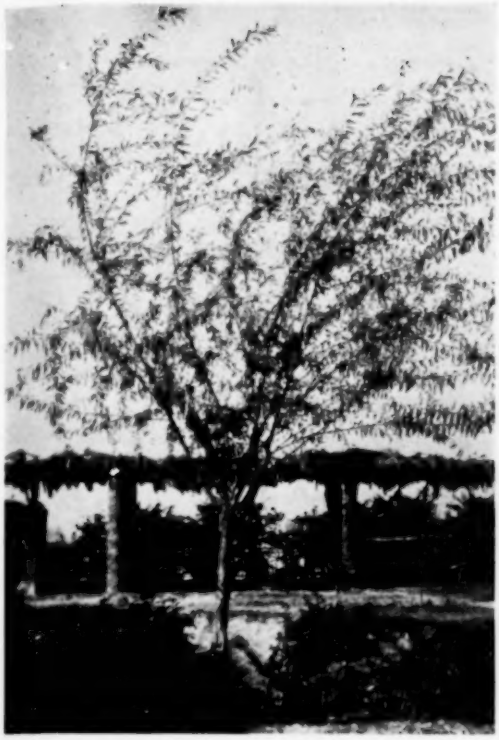

Fig. 2.—*Salix Safsaf* Tree. Medicinal Plant Garden, College of Pharmacy, Fouad I University, Cairo, Egypt.

S. Safsaf were collected from Wady El-Arish, Egypt in 1924. Figure 5 shows enlarged diagrams of a female *S. Safsaf* flower and a male *S. tetrasperma* flower. The whole catkin is usually from 5 to 8 cm. long and a single flower is about 2 to 3 mm. long.

Propagation.—The three species produce no seeds and are propagated by cuttings.

Barks

Morphology.—The barks of the three *Salix* species are nearly identical morphologically. Barks of young branches of the three species are generally quilled when dried, smooth, about 2 to 3 mm. thick, brownish green and showing transverse lenticels as well as buds on their outer surfaces. The inner surface is longitudinally striated with very minute elongated pits due to the medullary rays appearing in tangential view. Barks of old branches or of trunks are usually chanelled or flat pieces, about 4 to 7 mm. thick, deep brown in color, except that of *S. Safsaf* which is buff colored. The outer surface of the old barks is longitudinally fissured and transversely cracked. Odor is slightly aromatic; taste astringent and bitter except old barks of *S. tetrasperma* which are usually free of bitterness. Fracture is short on the outer side and fibrous on the inner.

Histology.—The barks of the three species are nearly identical in structure, but differ slightly in the dimensions of some corresponding elements and cell contents.

Salix babylonica Bark.—The cork is formed of few layers of tabular cells, with their outer tangential and radial walls very thickened and suberized, while their inner tangential walls are thin, having narrow lumina or mostly obliterated. In surface view, they appear polygonal, thick walled and dark yellowish brown. The phelloderm cells are very narrow, colorless, thin walled or equally thickened and not suberized.

The cortex is proportionally narrow. Its outer portion is collenchymatous, while the inner is parenchymatous. The pericycle is not quite distinct, and is represented only by few isolated groups of fibers.

The phloem, Fig. 6 (upper section), is composed of tangential groups of bast fibers which appear in transverse sections, alternating with bundles of soft bast, thus causing the stratification of the phloem. The bast fibers are slightly lignified, with thick walls and narrow lumina. They are accompanied by crystal sheaths, which contain prismatic calcium oxalate crystals embedded in thin membranes. The medullary rays are one cell thick and from 6 to 20 cells in height. Calcium oxalate rosette crystals and abundant minute ovoid starch grains are scattered throughout the parenchyma of the bark. Stone cells are totally absent from this bark.

Salix Safsaf Bark.—Histologically, this bark differs from *S. babylonica* bark in the dimensions of the elements and in the presence of groups of lignified, thick-walled, and narrow-lumened stone cells ensheathed by prismatic calcium oxalate crystals in the cortex of the former.

Salix tetrasperma Bark.—This bark resembles *S. Safsaf* bark in having stone cells. They only differ in element dimensions.

Fig. 3.—*Salix tetrasperma* Tree. Medicinal Plant Garden, College of Pharmacy, Fouad I University, Cairo, Egypt.

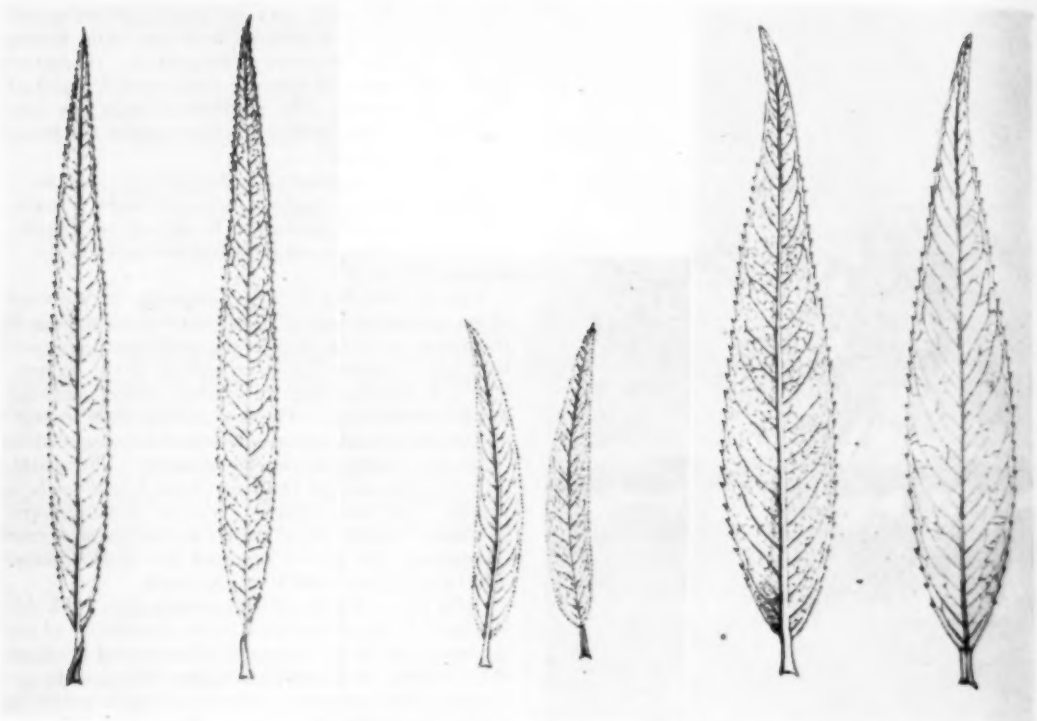

Fig. 4.—From left to right are shown diagrams of the lower and upper surfaces of the leaf of *S. babylonica*, the leaf of *S. Safsaf*, and the leaf of *S. tetrasperma*.

Conclusions and Summary.—Table I summarizes the average dimensions, in microns, of the elements and cell contents, as well as the average number of medullary ray cells indicating the thickness and height of their groups, in each of the three barks. The data given are the averages of at least fifty determinations for each element or cell content. From the table, the following conclusions may be derived:

1. Stone cells: Their absence is rather significant in differentiating *S. babylonica* bark from the other two barks, even in powder form.
2. Starch grains: Their size may help in differentiating the three barks.
3. Bast fibers: The thickest bast fibers are in *S. tetrasperma* bark, while the thinnest are in *S. Safsaf* bark.
4. Other elements: The calcium oxalate crystals, cork cells, crystal-sheath cells and the length of bast fibers cannot be relied on for the differentiation of the barks, since the ranges of their dimensions are overlapping and almost identical.

Leaves

Morphology.—From the comparative study of the morphological characters of the leaves of the three *Salix* species, it may be concluded that:

1. The three *Salix* leaves are simple, cauline, alternate, short petiolated and stipulate; the stipules being caducous.
2. The leaf lamina is simple, long, narrow lanceolate in *Salix babylonica* and *S. Safsaf;* but broadly lanceolate in *S. tetrasperma* leaves (Fig. 4).
3. The apex of the leaves of each species is pointed, acuminate, and generally discolored and broken.
4. The leaf of *S. babylonica* is unequal at the base; a distinction from the other two leaves.
5. The margins of the leaves of all species are generally serrate, but rarely entire in *S. Safsaf* and *S. tetrasperma*. The margin of the latter leaf generally becomes revolute towards the lower surface on drying. Each serration of the margin of the young *S. Safsaf* and *S. tetrasperma* leaves bears a conical parenchymatous projection which falls as the leaf becomes old.
6. The average lengths and maximum breadths of the leaf laminae of each species are recorded in Table II. It is clear from these data that the three leaves can be readily differentiated by size. Those of *S. Safsaf* are the shortest. They are about as half as long as those of the other two species. *Salix babylonica* leaf is the narrowest. Its breadth is less than half the breadth of the *Salix tetrasperma* leaf.
7. The Leaf-Index is the result of dividing the length of each lamina by its maximum breadth. Table II also shows the range of the Leaf-Index as well as its average for the mature leaves of each of the three *Salix* species. It is very clear from the table that the Leaf-Index is a reliable characteristic for differentiating the entire mature leaves of the species.
8. The upper surface of the leaves of each species is dark green, while the lower is paler and grayish green.
9. The laminae of the leaves of all the species are traversed by reticulate veins, which anastomose

TABLE I.—AVERAGE DIMENSIONS IN MICRONS OF ELEMENTS AND CELL CONTENTS OF *Salix* BARKS

Elements and Cell Contents	*Salix babylonica*, Microns	*Salix Safsaf* Microns	*Salix tetrasperma*, Microns
Stone Cells			
Diameter:			
Quadrangular	Absent	30 × 22–60 × 45	37 × 18–93 × 37
Elongated	Absent	52 × 26	82 × 30
Width of lumen:			
Quadrandular	Absent	18 × 15–48 × 33	22 × 15–35 × 23
Elongated	Absent	41 × 11–50 × 12	37 × 7–67 × 7
Bast Fibers			
Length	600–1600	800–1500	800–1850
Thickness	11–22	9–19	15–30
Width of lumen	2–13	2–11	7–16
Crystal-Sheath Cells			
Width	11–26	11–15	18–26
Height	15–18	7–10	11–18
Calcium Oxalate Crystals			
Prisms, diameter	11–18	6–15	8–22
Rosettes, diameter	15–45	11–30	19–45
Starch Grains, Diameter	2–5	4–8	4–11
Cork Cells, Diameter	18 × 11–56 × 18	15 × 15–49 × 18	18 × 15–41 × 18
Medullary Ray Cells, Number			
of:	cells	cells	cells
Thick	1	1	1
High	6 to 20	6 to 20	6 to 19

TABLE II.—RANGES AND AVERAGES OF LEAF-LAMINAE DIMENSIONS AND LEAF-INDEXES

Leaf	Length, Cm.	Maximum Breadth, Cm.	Leaf-Index	
S. babylonica	5–18	0.5–1.5	6.333–13.750	8.984 Av.
S. Safsaf	6–10	0.8–2.1	3.889– 7.308	5.805 Av.
S. tetrasperma	12–20	2.5–3.0	3.081– 4.700	4.105 Av.

near the margin. Dead veinules extend into the margin toward its serrations, one for each tooth but not reaching its apex. The midrib is more prominent on the lower surface; but the veinlets are more conspicuous on the upper surface. The veinlets, in the case of *S. babylonica* and *S. Safsaf*, leave the midrib at an angle between 35° and 45°; while in *S. tetrasperma*, this angle varies from 40° to 50°.

10. Both *S. babylonica* and *S. Safsaf* leaves are thin and papery when fresh; but when dry, they are brittle and easily broken and powdered between the fingers. The dried *S. tetrasperma* leaves are hard and coriaceous.

11. Salix leaves have a faint aromatic odor and a bitter taste. *Salix Safsaf* leaves are the most bitter.

Histological Characters Common for the Three Leaves

Histologically, the leaves of all the three *Salix* species are nearly identical in structure.

Petiole.—The lower part of the petiole is found to contain three vascular strands, each of which is composed of an arc of xylem, another of phloem and a small medullary parenchyma, all being included in a ring of pericyclic fibers. Travelling through the petiole upwards to the lamina, the three vascular strands tend to become rolled up, each forming a concentric structure. Then, they gradually fuse together and ultimately unite near the apex of the petiole into one vascular strand formed of two arcs, each of xylem and phloem, enclosing the lenticular body of medullary parenchyma and surrounded by an interrupted ring of pericyclic fibers. Calcium oxalate rosette crystals are scattered in the cortical tissue, but are more numerous near the pericyclic fibers.

Lamina.—Under this heading, the histology of the epidermises, the midrib, and mesophyll of the leaves will be discussed.

Epidermises.—The epidermal cells of the leaves are closely identical in shape, being tabular, hexagonal, and almost equal in size. Hence, they cannot be used as a differentiating character for the leaves. Some epidermal cells contain mucilage deposited generally on their inner walls and usually on their outer walls as well (8). The cuticle is somewhat thick and striated on the upper but not on the lower epidermis. Few stomata occur on the upper epidermis, but occur numerously on the lower. The stomata are of the "Rubiaceous type." The epidermis of the old leaves is devoid of hairs, while that of the young leaves bears few nonglandular unicellular hairs, which vary in length and width of lumen.

TABLE III.—AVERAGE DIMENSIONS, IN MICRONS, OF ELEMENTS AND CELL CONTENTS OF *Salix* LEAVES, THEIR VEIN-ISLET NUMBERS AND PALISADE RATIOS

Elements and Cell Contents	*Salix babylonica*, Microns	*Salix Safsaf*, Microns	*Salix tetrasperma*, Microns
Vessels, diameter	7–26	15–33	15–45
Pericyclic Fibers			
Length	1100–2700	500–1600	1300–3200
Thickness	7–15	9–19	19–26
Lumen, width	4–11	4–11	8–15
Epidermis, Lower			
Normal			
Width	26 x 22–90 x 34	19 x 19–56 x 37	11 x 45–75 x 38
Height	11–19	7–19	26–52
Neural			
Width	22 x 19–75 x 30	56 x 11–67 x 18	15 x 22–75 x 26
Height	11–19	7–19	7–15
Epidermis, Upper			
Normal			
Width	18 x 22–75 x 38	30 x 30–56 x 45	22 x 42–90 x 45
Height	11–19	11–26	26–52
Neural			
Width	11 x 24–45 x 26	12 x 30–75 x 19	30 x 11–75 x 15
Height	11–17	4–15	4–11
Hairs			
On Upper Epidermis:			
Length	600–1500	300–550	700–1300
Thickness	11–15	9–15	20–30
Lumen, width	8–11	8–13	15–23
On Lower Epidermis:			
Length	700–1500	1000–1400	550–1400
Thickness	11–15	15–19	20–30
Lumen, width	8–11	2–4	2–3
Calcium Oxalate:			
Prisms, Diameter	8–19	8–19	11–30
Rosettes, Diameter	11–22	11–22	15–37
Palisade Ratio	7–7.5	6.7–7.5	7.4–7.7
Vein-Islet Number	7.5	16	10.8

Hypodermis.—The lowest cell layer of the mesophyll is devoid of chlorophyll and is differentiated like a hypoderm, the cells of which are abutting on and alternating with those of the lower epidermis. Whereas, beneath the upper epidermis, no hypodermis exists.

Mesophyll.—It is heterogeneous and is composed of palisade and spongy tissues. The palisade tissue is not continuous through the midrib. In the interneural portions of the lamina it is composed of 4 to 5 cells in height and extends continuously from beneath the upper epidermis to the hypodermis of the lower surface. The spongy tissue is very scanty.

Midrib.—The midrib shows two arcs of vascular strands, and a central pith, Fig. 6 (lower section). Abutting on the outer side of each phloem arc is an arc of pericyclic fibers sheathed by prismatic crystals of calcium oxalate.

Calcium Oxalate.—Prismatic crystals occur only ensheathing the pericyclic fibers; while rosette crystals are scattered in the cortical tissue of the midrib and in the palisade tissue.

Histological Characters Differentiating the Three Leaves

The following characteristics may be of great help in the histological differentiation of the leaves either in the entire or powdered form:

Dimensions.—The difference in the dimensions of the respective elements or cell contents of the *Salix* leaves is probably the most reliable histological character for the differentiation of any leaf from the other two. Table III summarizes the average microscopical dimensions in microns as well as the "Palisade ratio" and "Vein-islet number" for each leaf.

Hairs.—They are nonglandular, unicellular, and occur on the epidermises of young leaves only. These hairs differ in the thickness of walls, width of lumen, and in length. The hairs on the upper epidermis of the leaves of all the species are thin walled, with wide luminae; the widest lumen is that of *S. tetrasperma* hairs. The hairs of *S. Safsaf* are the shortest, while those of the other two species are equal in length. Hairs on the lower epidermis of all the leaves are nearly equal in length, but those of *S. babylonica* are thin walled and have wide lumen. Those of the other two species are thick walled and narrow lumened.

Water Pores.—One and, rarely, two water pores are present on the upper epidermis of each tooth of *S. Safsaf* leaf margin, but totally absent from the other two leaves.

Vein-Islet Number.—It is a valuable differentiating character (9).

Cuticle Striations.—They are more distinct in the case of *S. tetrasperma* leaves.

Powdered Leaves.—The powder of any mature leaf of the three *Salix* leaves is very closely identical microscopically with that of the other two. The only significant difference lies in the dimensions of the respective elements as shown in Table III. However, if the powder includes that of very young leaves, the presence of their characteristic hairs will be a reliable feature for the identification and differentiation of the powdered leaves.

The powder of any of the leaves of these *Salix* species, is characterized microscopically by:

1. The mucilaginous epidermal cells with striated (upper), or smooth (lower), cuticle and rubiaceous stomata.
2. The spiral or pitted vessels and the crystal-sheathed pericyclic fibers.
3. The nonglandular unicellular hairs (if the powder includes that of very young leaves) or their scars.
4. The absence of any glandular structures.
5. Presence of the prismatic and rosette crystals of calcium oxalate.

CHEMICAL STUDY

The chemical investigation of the leaves and barks of the three *Salix* species was carried out with the object of isolating, identifying, and determining the yield of their active bitter principles.

Isolation of the Bitter Constituent

Principle.—The powdered barks or leaves are extracted with boiling alcohol. The alcohol is removed from the extract. Water is added and the mixture is purified by lead salts. The final purification of the yield is done by fractional solution followed by fractional precipitation and subsequent recrystallization until constant melting points are obtained. The whole process of extraction is carried out as rapidly as possible. Boiling and evaporation should be accomplished under reduced pressure, in order to avoid decomposition. Acid and enzyme hydrolysis must be guarded against.

Purification.—The bitter soft extract thus obtained, was decolorized and dissolved in the least possible amount of boiling absolute alcohol, then ether was added until turbidity occurred. Then the container was stoppered and refrigerated overnight. This fractional precipitation was continued until the addition of more ether to the decanted clear alcohol-ether liquid caused no more turbidity. Then petroleum ether was added instead of the ether. The obtained bitter crystalline or amorphous precipitates were further purified by repeated fractional solution and precipitation. Finally, the obtained product was recrystallized several times from alcohol until the melting point was constant.

Analysis of Isolated Bitter Constituent.—The purified isolated bitter substances were subjected to several tests for detecting their chemical nature. From the obtained results we can conclude that the bitter constituent of the barks and leaves of Salix species grown in Egypt is not an alkaloid or a bitter principle but a glycoside free of nitrogen, sulfur, and phosphorus.

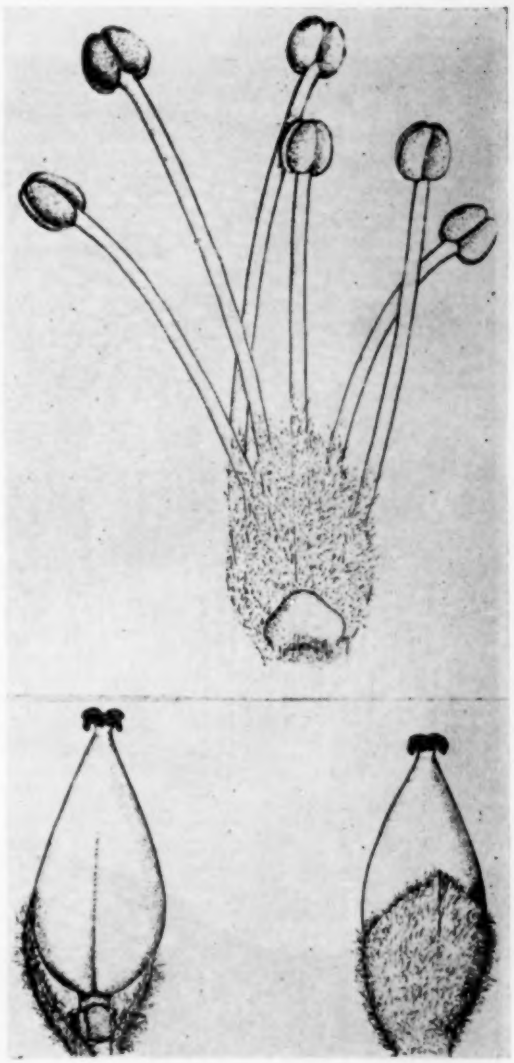

Fig. 5.—The upper diagram shows a ventral view of a single male flower of *Salix tetrasperma* from a catkin including the hairy bract, six stamens, and the nectar disk. The lower diagrams are a ventral view (left) and a dorsal view (right) of a single female flower of *Salix Safsaf* from a catkin showing the ovary, two bifid stigmas, a hairy bract, and a nectar disk.

It was proved by all physical and chemical tests that the bitter constituent of the barks and leaves of the three Salix species grown in Egypt is the pure glycoside "salicin," $C_{13}H_{18}O_7$.

Salicin Yield.—Salicin can be easily and accurately estimated either polarimetrically (10) or gravimetrically or colorimetrically (11), when in a more or less pure state, whether as powder or in solution. However, this is not the case when dealing with purified aqueous extracts of Salix barks and leaves, since they usually contain polysaccharides and other, probably glycosidal, coloring matters which are hydrolyzable by enzymes like emulsin, and by acids. Consequently, the only reliable

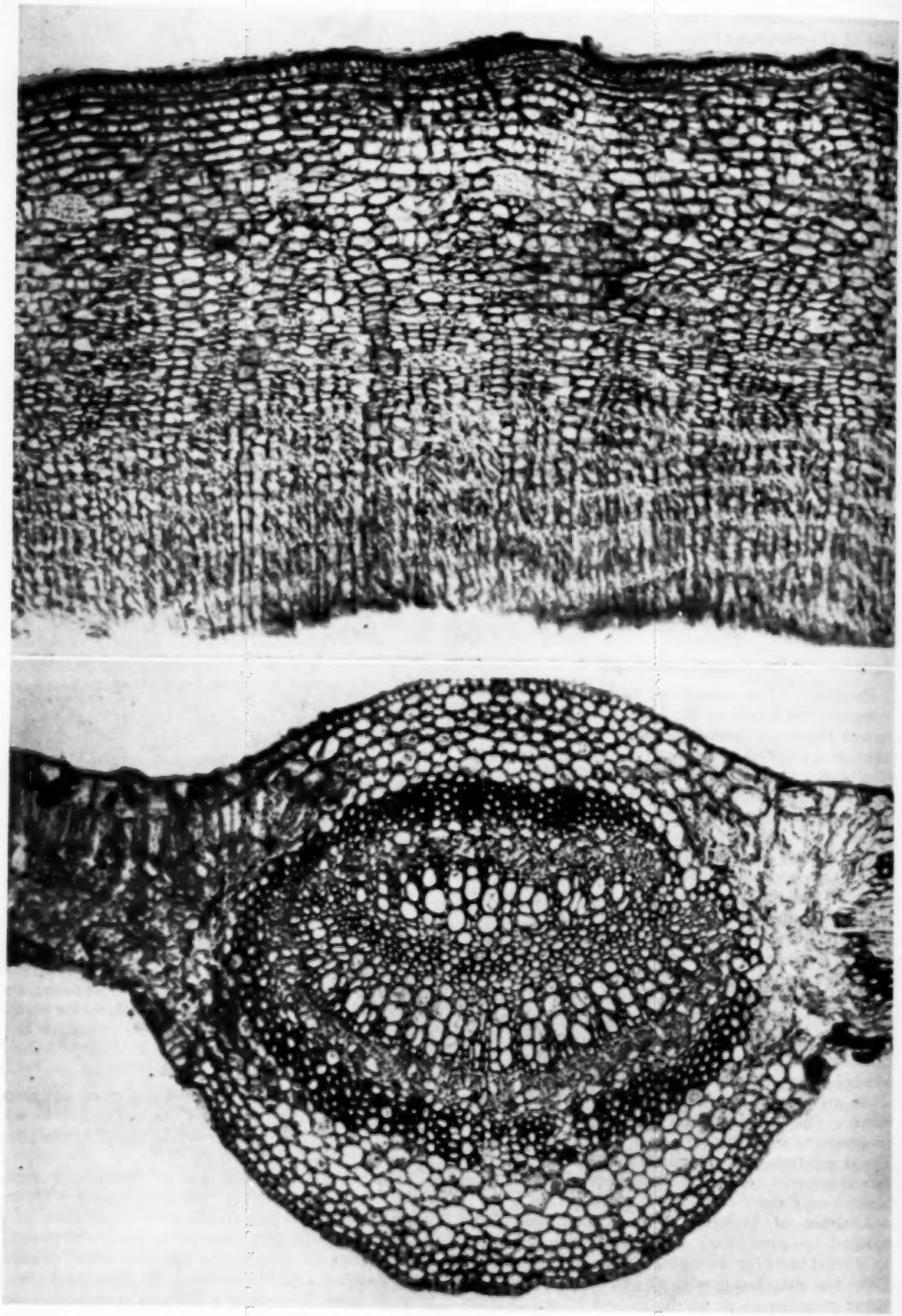

Fig. 6.—Transverse section of *Salix Safsaf* bark of a young branch (above) and transverse section of *Salix tetrasperma* leaf (lower section).

TABLE IV.—AVERAGE PERCENTAGES OF SALICIN YIELD FROM DRIED SALIX BARKS[a] AND LEAVES

Species	Per cent Salicin Yield from Barks	Leaves
Salix Safsaf	0.246	0.552
Salix babylonica	0.126	0.213
Salix tetrasperma	0.095	0.140

[a] The barks mentioned in the table are one to three years of age. No salicin could be isolated from the samples of older barks of any of the three species.

method of estimation and determination of the yield of salicin from the barks and leaves is its quantitative isolation. On the other hand, this method does not give the exact amount of salicin actually present in the extracted sample, since an appreciable amount of the glycoside may be lost during the long steps of the isolation and purification processes.

Table IV shows the average percentages of the yield of salicin from the dried barks and leaves of the three *Salix* species.

SUMMARY

1. The Egyptian *Salix* species are: *Salix babylonica* (Linné), *S. Safsaf* (Forsk.), and *S. tetrasperma* (Roxb.). They have been grown since the days of the Ancient Egyptians.

2. A complete taxonomical description of the trees is discussed.

3. The comparative morphological characteristics of the leaves and barks have been fully described. The barks are practically identical, while the leaves can be readily distinguished even by size.

4. Histologically, the barks are quite identical except for the presence of stone cells in both *S. Safsaf* and *S. tetrasperma* but not in *S. babylonica*. The barks also differ in the micro-dimensions of their respective elements, especially the bast fibers and the starch grains. The leaves are nearly identical in structure, differing only in the micro-dimensions of their respective elements, especially those of the pericyclic fibers, the diameter of vessels, and the width of lumen as well as the length of the nonglandular hairs (which only exist on the younger leaves).

5. While the Palisade ratios cannot be used as a character for the differentiation of the leaves since they overlap, the Vein-islet numbers are rather specific and are a valuable characteristic for the differentiation of the leaves.

6. The active bitter constituent of the barks and leaves has been isolated by the authors in a pure crystalline form and was proved to be the glycoside salicin, $C_{13}H_{18}O_7$. The amount of salicin extractable by the quantitative isolation from the leaves and barks ranged from an average of 0.095% in *S. tetrasperma* barks up to an average of 0.552% in *S. Safsaf* leaves. Barks older than three years of age were found almost free of salicin.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

D-Ascorbic Acid
L-Araboascorbic acid
L-Glucoascorbic acid
D-Galactoascorbic acid
Dehydroascorbic acid
Isoascorbic acid
Myristicin
Meconic acid
Geranic acid
3,5-Diiodothyronine
Thymine desoxyribose phosphoric acid
Cytosine desoxyriboside
Hypoxanthine desoxyribose phosphoric acid
Uridine
Xanthylic acid
Inosinic acid
Agmatin
Spermidine

Studies on the Leaf of *Salvia Leucophylla**

By HEBER W. YOUNGKEN† and E. WARREN HEAPS, JR.‡

A report of the results of an investigation of the leaves of a wild-growing California Sage which were offered on the market during the early part of World War II and were determined by the senior author as *Salvia leucophylla* Greene. This paper includes a pharmacognostical description of the physical characteristics of the leaves, the histology of the leaf blade and petiole and an initial report on the volatile constituents of the leaves.

DURING the early part of World War II there appeared on the market the dried leaves of a wild California Sage which was offered as a substitute for Sage. A sample of this material was submitted to the senior author for identification and determined to be the leaves of *Salvia leucophylla* Greene by comparison with herbarium material obtained from the University of Southern California, the Gray Herbarium, and the College herbarium. Since search of the literature failed to reveal any pharmacognostical work having been reported on the leaves of this species of *Salvia*, and since there appeared a possibility of some future economic use for this product owing to its aromatic and camphoraceous odor and taste, we investigated its physical and histological characteristics and some of the chemical aspects of its volatile constituents which are here reported for the first time.

METHODS

Study of Gross Leaves.—Seven pounds of the air-dried leaves, some attached to short segments of stem, were studied. They were spread out on the laboratory table and were carefully examined by means of a hand lens and by the usual organoleptic tests. Measurements were made by means of a metric rule.

Preparation of Sections.—Transverse sections of the petiole and leaf blade were prepared by the method of imbedding in paraffin. The whole dried leaf material was softened by maceration overnight in water and was then dehydrated by being passed through a graded series of alcohols, 10, 30, 50, 70, and 90%, and absolute, the material being allowed to remain one hour or more in each grade. The material was cleared by passing it through an alcohol-xylol series, allowing it to remain fifteen minutes or more in each grade. The series was: 3 alcohol to 1 xylol, 2 alcohol to 2 xylol, 1 alcohol to 3 xylol, xylol, xylol. The leaves were infiltrated with paraffin by adding solid paraffin to the xylol in which they were contained. After some hours at room temperature the corked vial was placed on top of an oven and more melted paraffin was added. The vial was uncorked to allow evaporation of the xylol, care being taken to avoid crystallization of the paraffin. The material in the xylol-paraffin mixture was then transferred to an open beaker and was placed in the oven to prevent solidification. It was then washed several times with pure melted paraffin and was allowed to stand one hour in the oven after each washing until free of xylol. The melted paraffin containing the leaf material was then poured into small cardboard boxes, the leaves placed in proper positions for later sectioning, and the blocks allowed to harden at room temperature. The selected leaf was cut from the paraffin block and transverse sections ranging from 10 μ to 20 μ in thickness were carefully cut with the Spencer Rotary Microtome. The sections embedded in the ribbons of paraffin were spread and fastened on slides, using albumen fixative (Mayer's) and low heat.

Staining of Sections.—The slides were placed upright in a well of xylol to remove the paraffin. They were transferred to equal parts of absolute alcohol and xylol, to absolute alcohol, 95, 70, 50, and 30% alcohol, successively, and then to water plus Safranin O, sufficient time being allowed to stain the lignified tissue present. The slides were then passed through a series as follows: water, 10, 30, 50, 70, and 95%, and absolute alcohol plus fast green, to stain non-lignfied tissue, then to absolute alcohol, absolute alcohol plus xylol, and xylol, successively.

The slides were removed from the xylol; xylol-balsam solution placed over the sections, and with cover slips carefully set in place were allowed to harden at room temperature.

Transverse sections were prepared also by cutting the leaf material held in halved elder pith and removing the sections, as cut by a Spencer Sliding Microtome, with a camel's hair brush. The sections thus cut, 10 μ to 25 μ in thickness, gave an excellent opportunity to study the non-glandular and glandular hairs present on the leaf epidermis, many of which were lost in the paraffin-prepared sections. The sections cut on the sliding microtome were stained by passing them through the series of alcohol, water, and stains, as described in paraffin sectioning. The stained transverse sections of the leaf in xylol were mounted permanently in xylol-balsam solution.

For surface sections of the leaf epidermis to be obtained, it was necessary to warm the leaf material on a watch glass in a 15% solution of chloral hydrate. This process softened the tissues sufficiently to allow the lateral pressure exerted between a slide and a cover slip on the leaf material to separate the epidermal tissue for study. Using a 50% solution of glycerin in water as a mounting medium, temporary mounts were prepared for study. Histological measurements were made by the use of a standardized ocular micrometer.

* Received October 1, 1947, from Massachusetts College of Pharmacy, Boston, Mass.
Presented to The Scientific Section, A. PH. A., Milwaukee, meeting August 1947.
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The methods employed in the study of the volatile constituents are discussed in the latter part of this paper.

DESCRIPTION OF SPECIES

Salvia leucophylla **Greene** (*Audibertia nivea* **Benth.) Purple Sage. Wild California Sage.**—*Salvia leucophylla* is an undershrub native to California where it attains a height of about 4 feet. Its stems are woody below, herbaceous above and bear opposite, short-petiolate lanceolate to oblong-lanceolate leaves with white tomentose lower surfaces and dense capitate verticillasters of flowers with light purple corollas. Jepson (1) has described the plant in further detail and gives its distribution as "Mts. along the coast, 500 to 1500 feet, from San Luis Obispo Co. to the Santo Inez, Santa Monica, and San Gabriel mountains."

Fig. 1.—*Salvia leucophylla* Greene or Wild California Sage. ×⅛.

Description of the Leaves of *Salvia leucophylla* **Greene.**—Leaves opposite, short-petiolate, the mature ones averaging 4 to 7 cm. in length, very pubescent, grooved above; lamina lanceolate, from 2 to 4 cm. in length, and from 0.4 to 1.2 cm. in width; apex acute to obtuse; base cuneate to auriculate, occasionally slightly oblique; margin distinctly crenulate; upper surface finely rugose, with depressed mid-rib, silvery gray to a very light green in color; densely pubescent throughout; venation pinnate-reticulate, the reticulations being very small; lower surface silvery gray to light green in color, mid-rib and veins prominent and densely pubescent; texture very velvety; odor camphoraceous and aromatic; taste aromatic, camphoraceous, becoming slightly bitter.

Histology of the Lamina.—The lamina in cross sections possesses an undulate outline with prominent convexities on its lower face representing mid-rib and smaller vein regions.

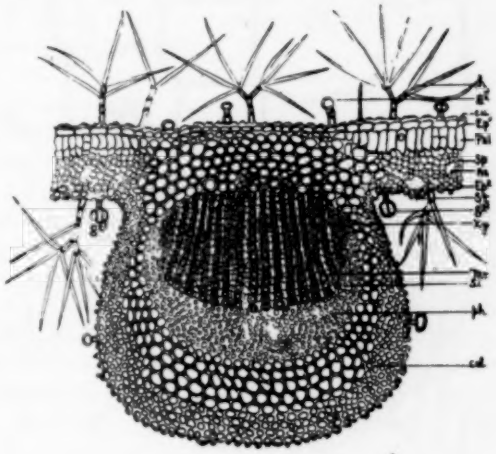


Fig. 2—Transverse section through the midrib and the interneural region of *Salvia leucophylla*

H, candelabra-shaped hair; *Ep*¹ upper epidermis; *sp*, spongy parenchyma; *Ep*², lower epidermis; *xy*, xylem; *si*, sieve tissue; *col*, collenchyma; *gl*¹, gland. hair, 1-celled head, 2-celled stalk; *gl*³, gland. hair, 3-celled head, 1-celled stalk; *cu*, cuticle; *pal*, palisade region; *as*, air space; *st*, stoma; *mr*, medullary ray; *ph*, phloem; *gl*², gland. hair, 2-celled head, 1-celled stalk.

Transverse sections of the interneural region of the leaf blade present for examination: (*a*) an upper epidermis of tangentially elongated epidermal cells whose outer walls are convex and are covered with a thick cuticle, whose outer portion is irregularly denticulate; (*b*) a palisade-parenchyma zone occupying one-third to one-half the width of the section, mostly one layer of cells in depth, but occasionally of two layers, the second layer of cells irregularly cuboidal to columnar; (*c*) a spongy parenchyma zone composed of small, irregularly shaped cells surrounding prominent air spaces and the bundles of veins, the latter containing spiral trachea; and (*d*) a sinuous lower epidermis composed of epidermal cells and stomata, the former being tangentially elongated with straight to convex outer walls. The lower epidermal cells are less than half as large as the cells of the upper epidermis, and they possess a thinner cuticle. The stomata occur in the sinus regions between the ribs. They are sunken in protruded regions of the lower surface. The epidermal cells adjacent to the stomatal orifice possess contents which stain red with Safranin O solution.

In surface section the upper epidermis shows polygonal to rounded polygonal epidermal cells, whose vertical walls are rectilinear to slightly curved.

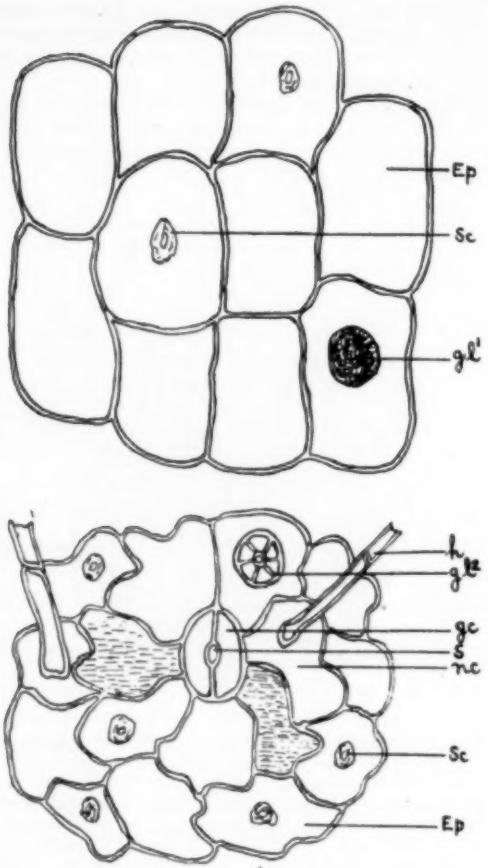


Fig. 3.—Surface view, upper epidermis, *Salvia leucophylla*

Ep, epidermal cell; *gl*[1], gland. hair, 1 celled head; *h*, non-glandular hair; *nc*, neighboring cell; *sc*, hair scar; *gl*[2], g land. hair, 6-celled head; *gc*, guard cell; *s*, stomatal opening.

Surface sections of the lower epidermis show sinuous-walled epidermal cells with many hair scars and hairs. The stomatal apparatus consists of two sunken guard cells surrounding the stomatal opening, which is quite small. The guard cells are in turn surrounded by two neighboring cells, which are perpendicular to the stomatal opening.

Both epidermal layers exhibit numerous glandular and nonglandular hairs. The nonglandular hairs are up to 306 μ in length and are of two types: (1) unicellular hairs and (2) multicellular hairs which are branched. The branched hairs exhibit varying kinds of branching. Some possess a unicellular to uniseriate axis from which two, usually unicellular, elongated, sharp-pointed branches emanate; others are candelabra-shaped. The glandular hairs are of the types shown in Table I.

The midrib region of the lamina is almost plano-convex in outline, showing a central crescent of open collateral bundles. The xylem regions consist of single radiating strands of vessels and tracheids, between which occur strands of narrow medullary rays, one to two cells in width. Collenchyma occupies most of the area between the bundles and the upper and lower epidermises. Both the upper and

TABLE I.—TYPES OF GLANDULAR HAIRS IN EPIDERMAL LAYER OF LAMINA OF *Salvia leucophylla*

Hair specimens	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7
Cells in head	6	1	1	2	2	6	1-2
Cells in stalk	0	1	2	1	2	1	3

the lower epidermises possess small, somewhat rounded epidermal cells, as seen in cross section, with convex outer walls covered with an irregularly denticular cuticle. Hairs similar to those described for the interneural region occur abundantly as outgrowths of both epidermises.

Histology of the Petiole.—The petiole in transverse section shows a concave upper surface and a convex lower surface. Both upper and lower epidermises of the leaf blade are composed of more or less rounded epidermal cells, whose outer walls are covered with a denticulate cuticle. In the central region of the petiole occurs a broad crescent of bundles consisting of radiating xylem strands, separated from each other by medullary rays, one to two cells in width. The phloem region of the crescent is flanked on its lower face by a zone of thick-walled, slightly lignified, pericylic fibers. Immediately surrounding the bundle region will be found parenchyma. A narrow zone of collenchyma occurs subjacent to each epidermis. Lateral to the broad crescent of bundles, both on the right and on the left, occur two small patches of vascular elements, which are bundle masses; thus there are five bundle strands coursing through the petiole. Hairs similar to those found on the lamina occur as outgrowths of both epidermises of the petiole.

VOLATILE CONSTITUENTS

Five separate 100-Gm. lots of the air-dried leaves of *Salvia leucophylla* were distilled, as directed in the U. S. P. XII[1] (2) and produced a solid which was deposited on and within the thimble of the water-cooled condenser. A light mineral oil bath and an electric stove were used. The temperature ranged from 105° to 110°. Approximately 95% of the solid was collected during the first hour of distillation, although each distillation was carried on for a period of five hours. The aqueous portion remaining in the distilling flask was treated with petroleum ether to remove volatile petroleum ether constituents, and was then evaporated. The products of this process were negligible.

The average percentage of solid distillate from 100 Gm. of leaves was 1.987. There was a variation of less than 0.3% by this method of distillation.

Since the solid substance had the characteristic appearance and odor of camphor, a melting point determination was made of the material obtained from each distillation. The melting points ranged from 162° to 168°. Purification of the solid material

[1] The method used in the distillation of the volatile constituents was that described in the United States Pharmacopœia XII, pp. 632–33. The melting point determinations were made by using the method described in the U. S. P. XII, pp. 595–97. The index of refraction was determined by the method described in the U. S. P. XII, p. 577. The specific gravity of the volatile oil was determined by the method described in the U. S. P. XII, pp. 576–77.

by recrystallization from ether, petroleum ether, and chloroform was carried out; the highest melting point obtained was 170.5°.

The melting point of U. S. P. camphor being 175° to 179°, the making of an oxime was carried out to substantiate the assumption that the solid obtained from the leaves of *Salvia leucophylla* was camphor.

The method of Gildermeister and Hoffmann (3) for the identification of camphor which was used with some modifications is as follows: Equal quantities of a 10% solution of the solid material in 95% alcohol and a 10% solution of hydroxylamine hydrochloride in 95% alcohol were heated on a water bath, and the same quantity of a 12% solution of sodium hydroxide was added; the resulting solution with its precipitate was heated on a water bath for a period of one hour. The oxime formed was precipitated by water, collected, and recrystallized from alcohol. It was necessary to purify the oxime by recrystallization from several solutions of petroleum ether. The solid material thus converted into the oxime, which has a melting point of 118–119°. The evidence seems good that the solid material obtained from the distillation of the leaves of *Salvia leucophylla* is largely camphor.

From the distillations of the 5 lots of the dried leaves of *Salvia leucophylla*, a light yellow volatile oil that was lighter than water was collected in the usual manner. The average percentage of volatile oil from 100 Gm. of dried leaves was 2.95. There was a variation of less than 0.3% by this method of distillation.

The index of refraction of the volatile oil was taken by the Abbé refractometer, five separate readings being taken at 26°. The average reading was 1.4650, with a mean variation of 0.0001.

The specific gravity of the oil was taken by a Sprengel pycnometer and was found to be 0.9102.

SUMMARY

1. The general characteristics and distribution of *Salvia leucophylla* Greene, as earlier recorded by Jepson, are cited.

2. A pharmacognostic description of the physical characteristics of the leaves of *Salvia leucophylla* Greene is given for the first time.

3. The histology of the leaf blade and petiole is discussed in detail and is illustrated by labeled drawings.

4. The most important diagnostically histological elements are the candelabra-shaped, branched, nonglandular hairs.

5. The methods used in the preparation of materials for histological study and for the determination of the volatile constituents of the leaves are described.

6. A volatile oil, lighter than water, was collected from distillations of five lots of dried leaves. The average percentage of volatile oil from 100 Gm. of dried leaves was found to be 2.95.

7. The following constants for the volatile oil of *Salvia leucophylla* Greene were determined: Sp. gr. 0.9102; refractive index, 1.4650 at 26°.

8. A solid substance having the physical characteristics of camphor was isolated from each of five distillates. The average percentage of solid distillate from 100 Gm. of *Salvia leucophylla* Greene leaves was found to be 1.987.

9. The melting point determinations of the crude solids isolated ranged from 162° to 168°.

10. By means of a modification of the method of Gildermeister and Hoffmann, the crude solid distillate was converted into the oxime (m. p. 118–119°); and the purified compound formed was found to have a melting point of 117.8°. Judging from the determined melting point of the oxime, it seemed evident that the solid distillate was in large part camphor.

REFERENCES

(1) Jepson, Willis L., "A Manual of the Flowering Plants of California," Associated Student Store, University of California, Berkeley, California, 1923–1925, p. 871.

(2) "The Pharmacopœia of the United States of America," Twelfth Revision, Mack Printing Co., Easton, Pa., 1942, pp. 632–33; 576–577; 595–97.

(3) Gildermeister, E., and Hoffmann, F. R., "The Volatile Oils," Tr. by E. Kremers, John Wiley & Sons, Inc., ed. 2, vol. 1, 1913, p. 457.

ERRATA FEBRUARY 1948 ISSUE

In the article entitled "Some Unsolved Problems in the Chemistry of Nucleic Acids," pages 45–53, the following corrections may be made:

Page 48.—In the formula for Diphospho-desoxypentosylcytosine change the double bond between carbon atoms no. 5 and no. 6 in the pyrimidine nucleus to a single bond.

Page 50.—In the second paragraph of the left hand column transpose "(Fig. 5)" to the end of the first sentence following the word "dissociations." Insert "(Fig. 6)" at the end of the third sentence and in the sentence following delete "as shown in Fig. 6."

Rapid Fluorometric Determination of Ketotetrahydropyridine Derivatives in Urine*

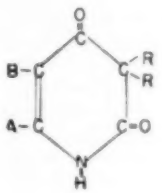
By ERICH HIRSCHBERG,† MARTHA FINE GREENBERG, ELMER DE RITTER, and SAUL H. RUBIN

A rapid method is described for the determination of 3,3-diethyl-2,4-diketotetrahydropyridine, a sedative-hypnotic, and related compounds in urine. It is based on the measurement of fluorescence in an alkaline, alcoholic medium before and after quenching with hydroxylamine. The accuracy and precision of the method are sufficient for application to clinical studies at dosage levels above 0.4 gram.

RENEWED clinical interest has recently been shown in an experimental sedative-hypnotic representative of the class of ketotetrahydropyridine derivatives. This drug, 3,3-diethyl-2,4-diketotetrahydropyridine, Persedon, was studied from the pharmacological point of view by Krautwald, Kuschinsky, and Riedel (1), and by Koppanyi, Herwick, Linegar, and Foster (2). These two groups of investigators also presented data on the urinary excretion of the drug by dogs and rabbits. The analytical method in the first study consisted of extraction of the drug from urine with chloroform, followed by evaporation of the solvent and weighing of the residue. In the second investigation, a colorimetric method developed by Koppanyi, Dille, Murphy, and Krop (3) for barbiturates was used to give an indication of the presence of Persedon in blood and urine.

The development of a rapid and more specific method of analysis for Persedon has recently been made possible by the finding of Kubli and Schmid (4) and Kubli (5) that compounds of the type shown in Fig. 1 exhibit an intense blue fluorescence in a strongly alkaline solution under ultraviolet light. The fluorescence is related to the shift of the keto-enol equilibrium toward the enol form in alkaline solution.

Simple measurement of the fluorescence of a solution of this drug suffices for the analysis of a pure solution or of a pharmaceutical preparation. In the assay of urine, however, appreciable extraneous blue fluorescence arises from other constituents so that supplementary treatment is needed to make the measurement more specific. A rapid method for the determination of Persedon and closely related compounds can be based on our observation that the fluorescence can be quenched quantitatively and selectively by hydroxylamine in the presence of normal fluorescent constituents of urine.



	R	A	B
I	C_2H_5	H	H
II	C_3H_7	H	H
III	C_2H_5	H	Br
IV	C_2H_5	COOH	H
V	C_3H_7	CH_3	H
VI	$CH_2 \cdot CHCH_2$	CH_3	H

Fig. 1.—Fluorescent ketotetrahydropyridine derivatives.

In this report, details of the method are given for Persedon and it is shown to perform in a satisfactory fashion in urinary excretion studies. Clinical data obtained in the course of such studies have been presented elsewhere (6–8).

METHOD

Reagents.—95% ethyl alcohol, redistilled. Hydroxylamine solution, 0.1 Gm. of $NH_2OH \cdot HCl$/ml. of H_2O. Sodium hydroxide solution, 10 Gm. of NaOH in 100 ml. of H_2O. Standard solutions: Stock, 75 mg. of 3,3-diethyl-2,4-diketotetrahydropyridine/100 ml. in 0.1 *N* H_2SO_4. Working increment solution, 11.25μg./ml.—subdilute stock standard 15.0 ml. to 100 ml. and again 5.0 ml. to 50 ml. in H_2O.

All reagents and standard solutions are stable for at least one month at room temperature.

Apparatus.—Fluorophotometer. In the present work, a Pfaltz and Bauer Model B, was used with the same filters as for thiochrome measurements, the primary ultraviolet filter having a peak of 3700 Å. and the secondary filters a peak of 4600 Å. A fluorescent glass block was used as a secondary standard to set the instrument.

Constant temperature bath, set at 75–80°.

Glass-stoppered tubes, 25–50 ml. capacity. Wire racks for holding tubes.

Procedure.—Since the most suitable galvanometer deflection (approximately 32 divisions at a glass block setting of 60 divisions) was obtained with the present fluorophotometer at a final Persedon concentration of 0.150 μg./ml., the dilution of

* Received Nov. 20, 1947, from the Nutrition Laboratories, Hoffmann-La Roche, Inc., Nutley, N. J.

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urine in water should contain about 0.5 μg./ml. Urine excreted in the first twenty-four hours after a normal dose of 0.6 Gm. of Persedon may usually be diluted 1 to 100. Although urine excreted during subsequent days normally contains low levels of Persedon, a dilution of 1 to 50 must be used to avoid measurement of an excessive amount of nonspecific fluorescence as detailed below. After suitable dilution, pipette 5.0 ml. of diluted urine and 10.0 ml. of alcohol into duplicate glass-stopped tubes. To one of the tubes, add 1.00 ml. of hydroxylamine reagent and to the duplicate tube 1.00 ml. of H_2O. Then add 0.8 ml. of 10% NaOH to both tubes. Set up two control tubes, one with hydroxylamine and one with water, containing 5.0 ml. of water in place of the urine sample. Thirty tubes plus two control tubes may be conveniently set up in one batch in wire racks.

Stopper all tubes and shake vigorously, loosen but do not remove the stoppers, and immerse the rack of tubes in a constant-temperature bath at 75–80° for fifteen minutes. At the end of the heating period, remove the racks of tubes and cool to room temperature in a cold water bath.

Take fluorescence readings as follows, using the glass block to set the fluorophotometer to a suitable galvanometer response (usually 60 divisions) just prior to each reading. Pipette 15.0 ml. of non-NH_2OH-treated sample into a cuvette and take the fluorescence reading (G); then pipette exactly 0.2 ml. of working increment solution of the standard into the cuvette, stir well, and take a second reading (I). For the blank reading (R), pipette 15.0 ml. of the NH_2OH-treated sample into a cuvette and read at the same instrument setting as for the corresponding unknown. Measure the fluorescence of 15.0 ml. of the non-NH_2OH treated control (B_G) and of the NH_2OH-treated control (B_R).

Calculation.—

$$\frac{(G - R) - (B_G - B_R)}{I - G} \times 0.15 \times \frac{D \times V}{1000} = \text{mg. of Persedon/day}$$

where D = total dilution of urine (168 and 336, respectively, for initial dilutions in H_2O of 1:50 and 1:100).

V = total volume of twenty-four-hour urine in ml., and other symbols are as stated above.

EXPERIMENTAL

Quenching of Fluorescence by Hydroxylamine.—It is evident that quenching of the fluorescence of the drug by hydroxylamine can be applied only if (*a*) the quenching is complete and (*b*) the fluorescence of other constituents of urine is not significantly affected by this treatment. That both conditions are fulfilled in the method presented is illustrated by the data in Table I. A typical, normal urine with and without the addition of 20 μg. of Persedon/ml. and a solution containing the same concentration of the drug in H_2O were diluted 3.0 ml. to 200 ml. and treated as directed. In pure solution, complete quenching of the fluorescence is observed. The effect of NH_2OH treatment on urine alone is relatively small; the average difference for 52 urine samples from 5 normal subjects was found to be 2.6 divisions (range 0.0 to 6.8) with an initial dilution of 1:100 in H_2O. The difference (38 divisions) between the total and blank fluorescence of the urine containing added drug, when corrected for the slight effect (2 divisions) of NH_2OH on the urine itself, is practically the same as the fluorescence of the drug in pure solution.

The amount of quenching of non-specific fluorescence depends on the dilution of the urine and the characteristic composition of the urines from different individuals. Table II shows the amount of nonspecific fluorescence and the extent of quenching by hydroxylamine at increasing concentrations of a normal urine. The per cent quenching is relatively constant over the entire dilution range, but at lesser dilutions than 1:150, the magnitude of the blank readings becomes excessive in relation to the fluorescence of normal concentrations of Persedon.

TABLE I.—EFFECT OF HYDROXYLAMINE

Solution	Fluorophotometer Reading[a]	
	Non-NH_2OH-treated	NH_2OH-treated
Drug in pure solution	37	0
Drug in urine	54	16
Urine alone	18	16

[a] Against setting of 100 with glass block and corrected for reagent blanks.

TABLE II.—EFFECT OF NH_2OH ON NONSPECIFIC FLUORESCENCE OF URINE

Over-all Dilution[a]	Total Fluorescence, G	Residual Fluorescence, R	% Quenching, $\frac{G - R}{G} \times 100$
1:500	8.0	6.0	25
1:300	15.0	11.0	20
1:250	19.0	16.0	16
1:200	23.0	17.0	26
1:150	28.0	23.0	18
1:100	44.0	31.0	30
1:50	80.0	57.0	29

[a] In alcoholic solution before addition of hydroxylamine solution (or water) and alkali.

Since a number of vitamins or their metabolites or oxidation products, including thiochrome, riboflavin, N^1-methylnicotinamide and 4-pyridoxic acid, are fluorescent, the possible influence of these compounds on Persedon assays has been investigated. Table III shows the effect of hydroxylamine treatment on a series of urines excreted in the course of availability tests in the twenty-four hours following a dose of 13.8 mg. of thiamine, 8.7 mg. of riboflavin, and 115 mg. of niacin. The fact that the magnitude of the fluorometric readings and the extent of quenching by hydroxylamine are essentially the same for these urines as for urines with normal vitamin levels at the same dilution, indicates that even high levels of thiamine, riboflavin, and N^1-methylnicotinamide do not interfere with Persedon assays. The fluorescence of 4-pyridoxic acid at normal urine concentrations would be insignificant at the dilutions employed in the present method.

It is possible, however, that greater interference may be encountered in some disease conditions. An adequate correction for the amount of nonspecific fluorescence quenched by hydroxylamine, both in

TABLE III.—LACK OF EFFECT OF HYDROXYLAMINE ON HIGH CONCENTRATIONS OF URINARY FLORESCENT VITAMINS

(At a 1:250 Over-all Dilution of Urine)

	Urinary Excretion Mg./Day			Fluorescence Galvanometer Divisions		
Subject	Thiamine	Riboflavin	N^1-Methylnicotinamide	Non-NH_2OH-treated	NH_2OH-treated	Difference
L. D.	1.83	5.1	14.6	21.4	18.5	2.9
F. W. J.	1.53	5.8	26.5	13.4	11.7	1.7
S. H. R.	2.44	8.0	19.5	25.4	23.8	1.6
J. C. B.	1.53	7.7	6.9	23.7	20.1	3.6
Mean	..	..	..	..	..	2.5

normal and pathological urines, may be made as follows. Twenty-four-hour collections of urine are made for several days or, preferably, a week before the first dose of the drug is given for an excretion study. These urines are assayed in the same manner and at the same dilutions as the subsequent urines containing the drug, and the average value obtained for "apparent drug" on these urines is used as a correction factor, i.e., subtracted from all subsequent excretion values. The daily values for this correction factor over the period of a week are fairly constant [e.g., 6.8 ± 3.3 (*S. D.*) mg./day of "apparent drug" were found in seven consecutive urines of a normal subject].

The effect of the concentration of urine on the magnitude of the blank (Table II) also provides a measure of the sensitivity of the method. Since a lesser dilution than 1:150 cannot be used, and since at least 0.05 μg. of the drug per ml. of final solution is needed for a suitable minimum deflection of the galvanometer (*cf.* Fig. 2), the lower limit of sensitivity is about 7.5 μg./ml. of urine (normally 5–10 mg./day). Since the average excretion of the drug after a dose of 0.6 Gm. ranges from 20–60 mg. for the first twenty-four hours, dropping to 10–25 mg. and 5–10 mg. on the second and third days (8), the analytical method is sensitive enough to assess the significant portion of the excretion curve.

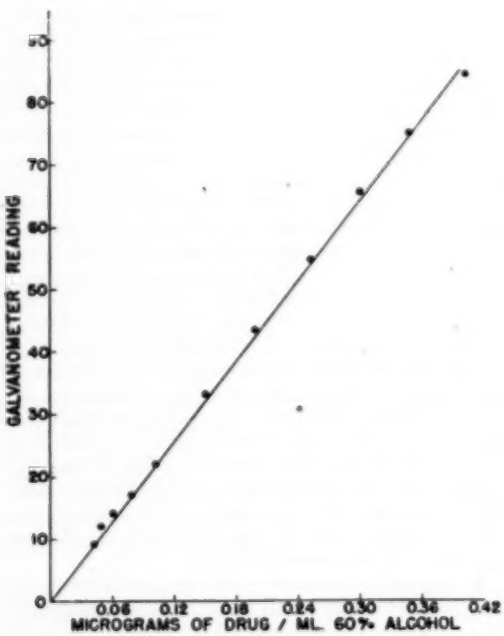

Fig. 2.—Calibration curve.

Relation of Fluorescence to Concentration.—The calibration curve (Fig. 2) at a glass block setting of 60 divisions, shows a linear relation between concentration of the drug and fluorescence in the range of 0.05 to 0.35 μg. per ml. Hence, in this range, the ratio of the fluorescence of the drug in the unknown to that of the increment is equal to the ratio of concentrations.

Effect of Alkali Concentration.—The relation of fluorescence of the drug to alkali concentration is shown in Fig. 3. A considerable amount of alkali is needed to drive the keto-enol equilibrium as completely as possible to the fluorescent enol form.

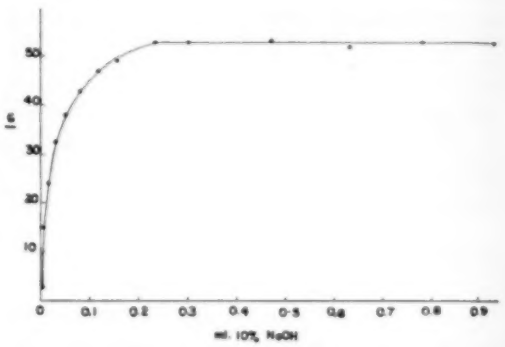

Fig. 3.—Effect of alkali concentration on fluorescence.

Effect of Concentration of Ethanol.—The influence of the percentage of alcohol in the final solution on the intensity of the fluorescence is shown in Fig. 4. The increase in fluorescence intensity with increasing alcohol concentration indicates the necessity for close control of this factor.

Stability.—A dilute aqueous solution of urine containing the drug is stable for at least four weeks at room temperature. The fluorescence in the final alkaline, alcoholic solution is stable for at least three hours. Heating for fifteen minutes at 75–80° has been found adequate for quenching the fluorescence with NH_2OH. The heating is limited to fifteen minutes to avoid possible concentration of solutions due to evaporation.

Recovery and Reproducibility.—The average recovery of the drug added to urine was 98 ± 4.4 (*S. D.*) per cent. The need for using an internal standard is shown by the fact that recoveries calculated against external standard readings were only 86 ± 4.0%, indicating appreciable quenching of the fluorescence. In view of the report by Ellin-

ger and Holden (9) that NaCl and other salts partially quench the fluorescence of thiochrome and riboflavin, it is pertinent to mention that, in the present case, no quenching occurred in the presence of fifty times (700 Gm./24-hour urine) the amount of NaCl found in normal urine.

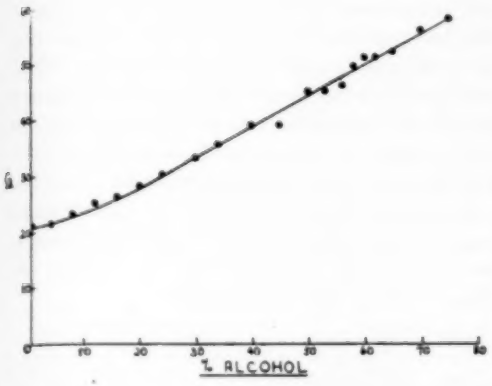

Fig. 4.—Effect of per cent alcohol on fluorescence.

A measure of the reproducibility of the method was obtained by carrying out duplicate or triplicate assays on seven normal urines containing 4 to 20 mg. of the drug per day. The average variation was 6.5%.

DISCUSSION

Several hundred normal and pathological urines have been assayed by this method during a period of a year. The simplicity and rapidity of the procedure are its principal advantages; the accuracy and precision are sufficient for application to clinical studies. The major difficulty encountered with the present method is its fairly low sensitivity, in relation to the urinary levels of the drug. The sensitivity is high enough to permit an accurate assay of urines following a dose of 0.4 to 0.6 Gm. of the drug, but reaches its limit at about the lower of these intake levels. The method is not sensitive enough for the analysis of blood levels. It may be necessary to introduce a concentration step (extraction or chromatographic adsorption) preliminary to the present method for the determination of lower levels of concentration.

While the main body of these data was obtained with 3,3-diethyl-2,4-diketotetrahydropyridine, other members of this group which exhibit appreciable fluorescence (4) (*cf.* Fig. 1) may also be determined in this way. Experiments with urines to which other members of the group were added indicated that in some instances the period of heating with hydroxylamine must be prolonged to obtain complete quenching of the fluorescence.

The mechanism of the reaction has not been completely defined. Hydroxylamine appears to behave here as an oxidizing agent (5, 10) with resultant disturbance of the conjugated double bond system, rather than as a ketone-addition reagent.

SUMMARY

A rapid and simple fluorometric method for the determination of 3,3-diethyl-2,4-diketotetrahydropyridine, a sedative-hypnotic, and related compounds in urine is based on the fact that their intense blue fluorescence is quenched quantitatively in the presence of normal constituents of urine by the action of hydroxylamine. The procedure is outlined and the relevant analytical conditions are discussed. The method is specific, precise, and sensitive enough for clinical excretion studies.

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A Study of Antacid Buffers. II. Prolonged Neutralization*

By J. M. HOLBERT, NANCY NOBLE, and I. W. GROTE

The results of a study of how long an antacid is effective in neutralizing gastric acidity under conditions simulating those in the stomach are reported. The technique involved consists of adding an excess dose of the antacid to a sample of artificial gastric juice. At various time intervals a portion of the mixture is removed and replaced with fresh gastric juice. The effectiveness of the antacid is followed by *p*H determinations. A comparison of ten antacids is given.

THE AUTHORS, in a previous paper (1), proposed an extension to the U. S. P. test for antacids, such as aluminum hydroxide, consisting of a method for determining both the extent of *p*H change and the time required for acid neutralization. Brief reference was made in this first paper to an article by the English workers, Johnson and Duncan, (2) in which the need for a time evaluation was stressed. The novel approach of these authors in testing the efficacy of antacids was of such interest that an application of their method, with certain variations, forms the basis of the present communication.

As was pointed out in our first paper, and in that of Johnson and Duncan, the mere neutralization of excess acid over a prolonged period of time is not a sufficient datum on which to judge the therapeutic value of an antacid. An efficient antacid should neutralize the gastric acidity rapidly to a *p*H of 3.5 to 4, thus performing a dual role in the prompt relief of pain and in the inactivation of pepsin. Actual alkalization of the gastric content by the use of a nonbuffering antacid is to be avoided since the use of such a material may lead to acid rebound, a condition in which the gastric mucosa responds to a temporary alkaline condition by the production of copious quantities of fresh acid (3). Slow acting antacids even though they do not produce a *p*H much greater than 4 are likewise of little value to the patient suffering from excess gastric acidity.

An additional point brought out by Johnson and Duncan concerns the length of time a given quantity of an antacid is effective in neutralizing acid. Such a test *in vitro* must simulate as closely as possible conditions present in the stomach; namely, the fresh secretion of acid and the periodic removal of portions of the gastric content. The method used by these workers consists first in the determination of the equivalent dose, which they define as the weight of the antacid in grams which will neutralize 100 ml. of 0.1 *N* HCl at 37.5° to *p*H 3.5 in fifteen minutes. The equivalent dose may be determined by trial and error or read directly from a curve of the neutralizing power of the antacid. The equivalent doses of several antacids as determined by Johnson and Duncan are given in Table I.

TABLE I.—EQUIVALENT DOSE

Antacid	Gm.
Magnesium oxide	0.23
Aluminum hydroxide gel	0.40
Calcium carbonate	0.57
Compound bismuth powder	0.62
Sodium bicarbonate	0.85
Magnesium phosphate	0.87
Aluminum hydroxide, powder A	1.20
Magnesium trisilicate	1.20
Calcium phosphate	1.35
Aluminum hydroxide, powder B	3.00
Aluminum trisilicate	5.00

In determining the length of time the antacid is effective in neutralizing gastric acidity, it is necessary that the dose of the antacid be in excess of that required to neutralize the acid present in the stomach at the time of administration. In this determination, the technique of Johnson and Duncan is to add twice the weight of the equivalent dose to 100 ml. of 0.1 *N* HCl at 37.5°. This mixture is rapidly stirred throughout the experiment and *p*H readings are taken electrometrically at various time intervals depending upon the rapidity of neutralization. At the end of ten minutes, 20 ml. of the reaction mixture is withdrawn to represent physiological loss from the stomach and 20 ml. of fresh 0.1 *N* HCl is added to the remaining mixture to simulate further secretion of gastric juice. This sequence of operations is repeated at ten-minute intervals until *p*H reading indicates that the neutralizing capacity of the antacid is depleted. A plot of time *vs.* *p*H allows a graphic interpretation of the efficacy of each antacid tested.

In our work we have modified this procedure of Johnson and Duncan in that we do not use twice the equivalent dose of the antacid, and have substituted artificial gastric juice for the 0.1 *N* HCl

* Received Sept. 27, 1947, from the Organic Research Laboratory, Brayten Pharmaceutical Company, Chattanooga, Tenn.

Presented to the Scientific Section, A. PH. A., Milwaukee meeting, August, 1947.

used by these workers. The use of the equivalent dose for determining the amount of antacid to be tested actually penalizes the rapidly acting antacid. For example, in the case of a rapidly acting antacid such as aluminum dihydroxy aminoacetate, 1 Gm. of which neutralized 150 ml. artificial gastric juice to *p*H 3.5 in five minutes, the equivalent dose as defined by Johnson and Duncan is so small that any prolonged neutralizing effect is impossible. Therefore, since our previous work was done with 1 Gm. of antacid tablet material in 150 ml. artificial gastric juice, we have used for the prolonged neutralization studies 2 Gms. of tablet material in 150 ml. of artificial gastric juice.

EXPERIMENTAL

Two grams of the finely ground tablet material is introduced into 150 ml. artificial gastric juice maintained at 38°. The mixture is efficiently agitated with a stirrer so that no settling of antacid material occurs. The *p*H of the mixture is determined by the glass electrode at intervals of one, three, five, and ten minutes. At the end of ten minutes, 20 ml. of the reaction mixture is removed and replaced with 20 ml. of fresh artificial gastric juice at 38°. This process is repeated at ten-minute intervals until the *p*H indicates that the neutralizing power of the antacid is spent. Table II gives the results of the investigation of ten well-known antacid materials.

duced an initial *p*H rise considerably above the desired 3.5 to 4. In the two curves 8 and 9, illustrating magnesium hydroxide antacids, the *p*H was actually on the alkaline side. In a few cases the antacid failed to control the *p*H long enough to be of appreciable value. Judging from curves 1 and 2, representing aluminum dihydroxy aminoacetate and a brand of aluminum hydroxide, it should be possible to control gastric acidity over prolonged periods of time by the proper dosage of these materials.

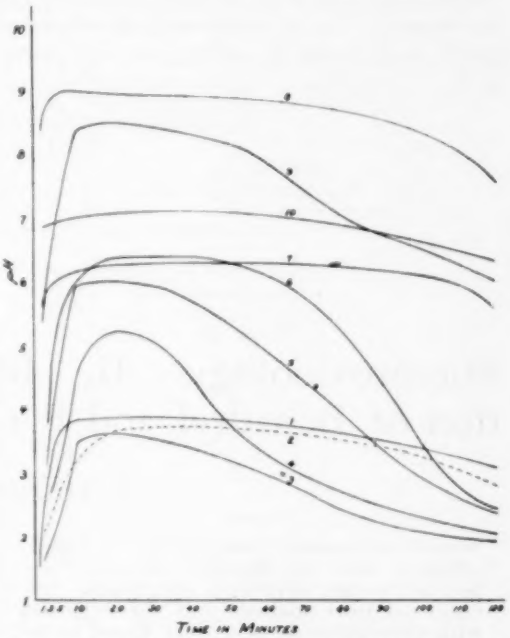

Fig. 1.—Graphical representation of data in Table II.

TABLE II.—A COMPARISON OF ANTACIDS BY A MODIFICATION OF THE JOHNSON AND DUNCAN TECHNIQUE

Time in Minutes	*p*H									
	I	II	III	IV	V	VI	VII	VIII	IX	X
0	1.56	1.62	1.60	1.57	1.70	1.62	1.62	1.68	1.58	1.70
1	1.98	2.12	1.67	1.64	2.33	2.00	5.62	8.40	5.40	6.89
3	3.14	2.33	1.90	2.14	3.62	4.33	5.95	8.92	6.40	6.93
5	3.62	2.53	2.32	3.05	4.80	5.38	6.06	9.00	7.18	6.94
10	3.85	3.20	3.49	4.44	5.98	6.06	6.21	9.04	8.40	7.03
20	3.90	3.70	3.64	5.26	6.03	6.43	6.27	9.00	8.54	7.11
30	3.91	3.74	3.54	5.10	5.97	6.43	6.33	8.98	8.49	7.15
40	3.88	3.73	3.40	4.50	5.77	6.43	6.33	8.96	8.38	7.16
50	3.84	3.71	3.21	3.73	5.49	6.33	6.32	8.92	8.22	7.10
60	3.78	3.68	3.00	3.29	4.98	6.16	6.33	8.88	7.97	7.03
70	3.73	3.63	2.71	2.98	4.50	5.82	6.30	8.81	7.47	6.95
80	3.64	3.52	2.41	2.72	3.97	5.18	6.28	8.73	7.08	6.87
90	3.54	3.45	2.24	2.50	3.40	4.35	6.22	8.63	6.78	6.76
100	3.41	3.30	2.10	2.32	2.96	3.50	6.16	8.40	6.59	6.66
110	3.28	3.09	2.03	2.19	2.65	2.84	6.02	8.13	6.34	6.52
120	3.10	2.83	1.96	2.07	2.40	2.42	5.63	7.65	6.03	6.38

Legend:

I. Aluminum dihydroxy aminoacetate.
II. Aluminum hydroxide, Brand A.
III. Aluminum hydroxide, Brand B.
IV. Aluminum hydroxide, magnesium trisilicate.
V. Magnesium trisilicate.
VI. Magnesium trisilicate, calcium carbonate.
VII. Magnesium trisilicate, calcium carbonate, magnesium carbonate.
VIII. Magnesium hydroxide.
IX. Magnesium hydroxide, calcium carbonate, bismuth subcarbonate, sodium bicarbonate.
X. Sodium bicarbonate.

Figure 1 represents the results in Table II graphically.

A study of the above curves shows that with the exception of the aluminum compounds, curves 1, 2, and 3, the use of an excess amount of antacid pro-

SUMMARY

1. Studies are presented illustrating the effect of excess doses of antacids upon artificial gastric juice.

2. These studies were carried out under conditions simulating those probably encountered in the stomach; that is, the intermittent removal of gastric content and the secretion of fresh gastric juice.

3. A comparison of ten well-known antacids is given.

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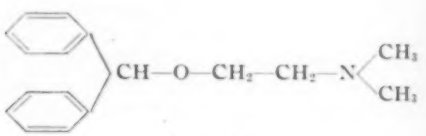
Microtoxicology. II. Identification and Differentiation of Benadryl and Pyribenzamine Hydrochlorides*

By THOMAS J. HALEY†

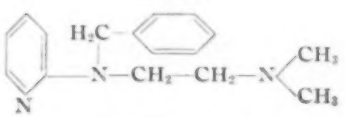
Several colorimetric differentiation and identification tests for Benadryl and for Pyribenzamine are described. Of twelve precipitation tests studied, only the reactions with chloroplatinic acid were found to be applicable to these compounds for purposes of identification and differentiation.

THE ANTIHISTAMINIC drugs, Benadryl Hydrochloride (Diphenhydramine Hydrochloride, N. N. R.) and Pyribenzamine Hydrochloride (Triplennamine Hydrochloride, N. N. R.), have recently come into wide usage in the treatment of allergenic conditions. Many reports of side reactions due to their use are already in the literature (1–13). Gelvin and McGavack (14) have reported a colorimetric method for the quantitative determination of Benadryl in body fluids. Keenan (15) has shown that both of these antihistamines may be identified by their reactions with chloroplatinic acid. Nonspecific methods for the identification and analysis of both drugs have been described in New and Nonofficial Remedies (16, 17).

In devising methods of identification it is necessary to observe the structural similarities and differences between drugs with similar action so that chemical identification may be made.



Benadryl



Pyribenzamine

From a chemical viewpoint Benadryl may be considered as an ethanolamine derivative and Pyribenzamine as an ethylenediamine derivative. Benadryl may also be considered as an ether or as a substituted diphenylmethane compound and Pyribenzamine as a benzyl or pyridine compound. It was upon this basis that the two antihistamines were tested with the general alkaloidal precipitants and colorimetric reagents as well as with general amine reagents and other reagents which are used to identify similar compounds such as Procaine.

EXPERIMENTAL

The alkaloidal precipitation and colorimetric reagents were prepared as directed by the references in Tables I and II. To one drop of the former on a microscope slide were added either a few crystals or one drop of a one per cent solution of either antihistamine compound. The colorimetric tests were carried out on a spot plate, a few crystals of the antihistamine being added to ten drops of the rea-

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gent. In those reactions which were done in the test tube, the required amount of reagent and a few crystals of antihistamine were mixed according to the usual procedure. The results of the precipitation reactions are given in Table I and those of the colorimetric reactions in Table II.

The similarity of the structure of Procaine to that of Benadryl would lead one to believe that the latter should react with the same precipitation reagents. Procaine forms irregular plates with one per cent sodium bromide solution but both Benadryl and Pyribenzamine were nonreactive with this

TABLE I.—PRECIPITATION REACTIONS OF BENADRYL AND PYRIBENZAMINE HYDROCHLORIDES

Reagent	Benadryl HCl	Pyribenzamine HCl	Reagent Reference
Chloroplatinic acid 5%	Granular orange precipitate. Leaf-like crystals in crosses. Cigar shape for some of the crystals	Granular orange precipitate. Rosettes and sheaves of flat plates on drying	15
Chloroauric acid 1:20	Granular yellow precipitate	Granular yellow precipitate	18
Phosphotungstic Acid	Amorphous white precipitate	Amorphous white precipitate	18
Silicotungstic acid 5%	Amorphous white precipitate	Amorphous white precipitate	18
Phosphomolybdic Acid 10%	Amorphous greenish precipitate	Amorphous greenish precipitate	18
Mercuric Chloride 1:20	Granular white precipitate	Granular white precipitate	18
Mayer's Reagent	Granular yellow precipitate	Granular yellow precipitate	18
Wagner's Reagent	Granular brown precipitate	Granular brown precipitate	18
Picric Acid Sat. Aqueous Solution	Granular yellow precipitate	Granular yellow precipitate	18
Sodium Bromide 1%	No reaction	No reaction	19
Potassium Ferrocyanide 2%	No reaction	No reaction	19
Copper Sulfate–Pyridine	No reaction	No reaction	19

TABLE II.—COLORIMETRIC REACTIONS OF BENADRYL AND PYRIBENZAMINE HYDROCHLORIDES

Reagent	Benadryl HCl	Pyribenzamine HCl	Reagent Reference
Mandelin	Red with oily red globules	Chocolate-brown	18
Marquis	Canary-yellow, reddish orange then chocolate-brown	Red then deep reddish brown	18
Mecke	Canary-yellow then reddish orange	Nut-brown	18
Frohde	Canary-yellow, orange then orange-red	Pale pink then rust-red	18
Conc. Sulfuric Acid	Orange	Greenish yellow	16, 17
Conc. Nitric Acid	No reaction	No reaction	18
Potassium Dichromate and Conc. Sulfuric Acid	Yellow	Brown	18
Benzaldehyde–Sulfuric Acid 1:1	No reaction	No reaction	20
Ferric Chloride 9%	No reaction	No reaction	21
Resorcinol and Conc. Sulfuric Acid	Orange then reddish orange. Wine color on dilution with water	Yellowish green then deep green. Olive-green on dilution with water	22
Diphenylamine	No reaction	No reaction	23
Furfurol and Sulfuric Acid	No reaction	No reaction	24
Furfurol 1% Overlay Sulfuric Acid	Orange-brown, yellow-green on shaking	Black, no change on shaking	25
Furfurol and Glacial Acetic Acid	No reaction	No reaction	26
Chromic Acid 5%	Orange-red precipitate	Orange precipitate	27
Foucry	Cherry-red	Cherry-red	28

DISCUSSION

Both Benadryl and Pyribenzamine gave the same type of precipitates with most of the precipitation reagents, thus showing that such reagents cannot be used for their identification or differentiation. However, with chloroplatinic acid definite crystalline precipitates were formed. These crystals were identical with those described by Keenan (15) and can be used to identify and differentiate the two compounds.

reagent. Similar results were obtained with the copper-pyridine reagent used to identify the barbiturates and with the potassium ferrocyanide reagent used to identify antipyrine.

The colorimetric reactions of both Benadryl and Pyribenzamine with many reagents are distinctive and differ from those recorded in the literature for other similar compounds. However, several reagents which react with other similar compounds are nonreactive with the antihistamines.

Mandelin's reagent can be used to identify and

differentiate the two antihistamines not only from each other but from other compounds which produce distinct colors with this reagent. The preliminary reactions of brucine and solanine are similar to Benadryl but both of the former show final colors which are distinctly different from the latter. The same is true for Pyribenzamine although the shade of color given by it and emetine are similar. However, other tests will aid in differentiating these two compounds.

Marquis' reagent gives different colors for the antihistamines than for such alkaloids as morphine, papaverine, and veratrine. However, the investigator must observe not only the first but the final colors to be certain of the exact compound causing them.

Mecke's reagent gives color reactions with colchicine and veratrine which in their early stages are similar to that given by Benadryl but the final colors are so different that no mistake can be made in differentiating the compounds.

Frohde's reagent gives a yellow color with both colchicine and veratrine but the final colors differ from the colors produced by Benadryl. There are no other compounds which produce a color which is similar to that given by Pyribenzamine.

The reaction of Pyribenzamine with concentrated sulfuric acid is very similar to that of berberine, narceine, and veratrine but simultaneous observation of the four compounds will aid in differentiating them. The other types of tests would aid in identifying the exact compound involved. One of the intermediate colors of veratrine is orange and this might be confused with the color given by Benadryl but the former compound gives a cherry-red as a final color while the color given by Benadryl remains the same. The difference in the reported color reaction for Benadryl (17) and that given in Table II may be due to interpretation of the end point. Complete destruction of an organic compound by concentrated sulfuric acid results in a dark brown to black solution which is not suitable for qualitative identification.

The dichromate test which is distinctive for strychnine gives a different color reaction with the two antihistamines so that they could not be mistaken for strychnine.

The resorcinol-sulfuric acid test for Lactophenin is very useful for differentiating the two antihistamines because the colors produced both before and after dilution with water are very different and differ from those given by Lactophenin.

Only one of the furfurol reagents reacted with these compounds but the test would enable the toxicologist to differentiate Benadryl from Pyribenzamine and also from Procaine. The latter compound gives a rose-red to purple-red color with this reagent. The results with the furfurol-acetic acid reagent confirm those obtained by Sanchez (26) in that the reagent gives a red, orange, or purple color only when there is a primary amine group attached directly to the aromatic nucleus.

With Woodward's chromic acid reagent both compounds respond with a color similar to that given by cocaine. Inasmuch as Procaine does not react with this reagent, the test could be used to differentiate between the antihistamines and that local anesthetic.

The reaction given by the antihistamines with Foucry's reagent is the same as that given by many other amines and thus is of no value in identifying these drugs.

SUMMARY

The reactions of twelve precipitation and sixteen colorimetric reagents with Benadryl and Pyribenzamine have been studied. The only precipitation reagent which is useful in identifying these compounds is chloroplatinic acid. Benadryl and Pyribenzamine gave distinctly different color reactions with most of the colorimetric reagents employed and these reactions are sufficiently different from those for other drugs recorded in the literature to prevent these antihistamines from being mistaken for other compounds.

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